comprising expression regulatory sequencesoperably linked to said exogenous gene encoding said protein or peptide, wherein said organic material is feces, urine, microbe, a chemical pollutant or a by-product thereof, a food product or a by-product thereof; and

(b) allowing said exogenous gene encoding said protein or peptide to be expressed and said protein or peptide to be secreted into the urine of said transgenic animal.

56. (Twice Amended) A non-human transgenic animal that produces in its urine a protein or peptide that degrades or detoxifies organic material, wherein said transgenic animal has stably integrated into its genome an exogenous gene encoding a protein or peptide that degrades or detoxifies organic material comprising expression regulatory sequencesoperably linked to said exogenous gene encoding said protein or peptide that is detectable in the urine of said transgenic animal, wherein said organic material is feces, urine, microbe, a chemical pollutant or a by-product thereof, a food product or a by-product thereof.

63. (Twice Amended) The transgenic animal of claim 72, wherein said kidney-specific regulatory sequence comprises a uromodulin promoter.

REMARKS

Upon entry of this amendment, claims 5, 7, 12-15, 45-47, 51-57, 61-65 and 67-74 are pending. Claims 6 and 11 have been canceled and claims 45, 56 and 63 are amended. Applicants have also amended claims 45 and 56 clearly define the phrase "organic material." The amendment to claim 63 corrects a typographical error. Acknowledgement is made of the withdrawal of the rejections under 35 U.S.C. § 112, second paragraph, 35 U.S.C. § 102 based on Sun *et al.* (U.S. 5,824,543) or Sun *et al.*(WO 96/39494) and 35 U.S.C. § 103 based on Sun *et al.* (U.S. 5,824,543) or Sun *et al.*(WO 96/39494) and Lubon *et al.*

1. Claim Objections

The Examiner objects to claim 63 as depending from claim 56 and 72 without indicating that these are in the alternative. This problem is a result of a typographical error in the previous response. Applicants have amended Claim 63 to indicate that it depends from claim 72.

2. Rejections under 35 U.S.C. 112, first paragraph

The Examiner states that claims 5-7, 11-13, 15, 45-47, 51-57 and 61-65 stand rejected and claims 67-74 are rejected under 35 U.S.C. first paragraph as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to make or use the invention commensurate with the scope of the claimed invention. Applicants will provide copies of the prior art discussed below in a supplemental response.

Applicants respectfully disagree with the Examiner and traverse the rejection. Applicants contend that the specification is enabling for the method of producing a protein or peptide in the urine of a transgenic animal wherein the protein or peptide degrades or detoxifies organic material. The specification provides sufficient disclosure and guidance for a skilled artisan to practice the present invention.

The Examiner states that the prior art has shown that mammary specific regulatory regions can reproducibly direct a protein of interest to the milk, blood or urine a representative number of transgenic mammals. Applicants should not be required to show that the invention works in all species. The skill of the artisan is high in the transgenic art and there is sufficient guidance from work with other species to prepare all of the claimed species of animals.

In response to the Examiner, applicants contend that it is known to those in the art, that mammalian promoters do function in species like birds and that correlative regulatory sequences exist and can be identified by those skilled in the art. For example, the chicken ovalbumin promoter, chicken beta-globulin genes have long been used in transgenic animals and plants (Taboit-Dameron et al., Transgenic Res 1999, 8: 223-235; Reitman et al., Nature 1990 Dec 20-27;348(6303):749-52). The CRABP-1 locus from chicken and puffer fish was expressed in mice, and both genes were shown to be expressed in the same subset of tissues as the mouse promoter (Kleinjan et al., 1998, Transgenic Res. 7: 85-94). The uromodulin gene was also found among all vertebrate classes (Badgett et al., Urol Int 61: 72-75, 1998). Uromodulin was detected in the skin of several amphibians and fish, in the oral mucosa and gills of fish, and the distal tubules of the kidney of some amphibians (Howie et al., Cell Tissue Res 1993 274(1):15-9). In addition to the human uromodulin gene which the Applicants isolated and sequenced, the bovine and rodent uromodulin genes and promoter have been cloned and characterized (Yu H et al., 1994, Gene Expr. 4: 63-75).

The Examiner states that the examples provided in Figure 5 and Table III of the present invention are limited to the production of transgenic mammals using the WAP promoter. Applicants respectfully disagree with the Examiner. The present application is not limited to the use of the WAP promoter since there is sufficient disclosure in the specification that provides regulatory elements and sequences other than the WAP promoter that can be used to express proteins in the urinary tract of the transgenic host. See pages 28 to 30 of the specification. Applicants disclose that the apolipoprotein E gene promoter, the uromodulin promoter and the uroplakin promoter are suitable regulatory sequences that are useful to drive expression of a protein that it is detectable in urine (see page 28 of the present application). Moreover, applicants specifically state that promoters suitable for use in preparing transgenic animals according to the present invention, particularly include the uromodulin and uroplakin promoters. See page 28, line 28 to line 32. Furthermore, Applicants describe the use of inducible promoters such as metallothionien promoters, as being suitable for the present invention. See page 29, line 32 to page 30, line 7.

The Examiner reports that the specification does not teach any 5' or 3' urinary tract-specific regulatory sequences that function to target protein expression to the urinary tract. Applicants respectfully direct the Examiner to the disclosure in the specification that states the urinary tract-specific translation regulatory sequences of the uromodulin, uroplakin, renin, erythropoietin, uropontin, nephrocalcin, aquaporin genes are preferred sequences that are useful in the present invention (see page 28 of the present application). Indeed, Example 3 (page 42) of the present application states the 5' and 3' sequences of uromodulin, uroplakin,renin, erythropoietin, apolipoprotein e, aquaporin, nephrocalcin, osteopontin-k / uropontin, urinary kallikrein and urinary thrombomodulin genes are used in the present invention. Applicants have isolated the uromodulin promoter, sequenced it and prepared constructs for expressing EPO in transgenic animals.

While the specification does not teach "any and all" gene promoters, the regulatory regions of many genes expressed in the urinary tract have been sequenced and are known or available to the skilled artisan. Applicants contend that the skilled artisan is sufficiently skilled to select, isolate, manipulate and prepare constructs containing appropriate 5' and 3' regulatory

elements of the described genes for use in preparing transgenic animals that express a specific protein or peptide that degrades or detoxifies organic material.

Applicants also do not define enzymes as those that degrade components of the extracellular matrix. Applicants define suitable enzymes, such as those that are pre-adapted to convert waste products, such as those enzymes present in microorganisms. Applicants respectfully disagree with the Examiner that the specification does not teach a skilled person how to identify proteins that degrade organic material in the context of the present invention. Applicants provide examples in the present application of particularly useful enzymes. In this regard, applicants suggest suitable enzymes and biologically active peptides or proteins as those which, for example, degrade penicillin antibiotics in excreta, affect the composition of the soil and/or surface and groundwater, or detoxify pesticides contaminating soil and water. See page 18, line 31 to page 19, line 9 of the present specification.

The Examiner expresses concern that synthesis of enzymes that degrade organic materials would be detrimental to the host organism or animal. Due to the Examiner's broad interpretation of "organic material," the Examiner again cites an unsuitable study (D' Armiento *et al.*) as evidence that expression of a matrix metalloprotein collagenase in mice has fatal consequences. Applicants respectfully point out that collagenase also degrades the extracellular matrix surrounding cells in all tissues of the body. In D' Armiento *et al.*, it was to be predicted that transgenic expression in the skin would cause health problems, because collagenase is expressed in most tissues that are undergoing remodelling, and also in several disease states, including rheumatoid arthritis and cancer invasion. Applicants wish to emphasize again that proteins, such as collagenase and stromelysin-1, are not typical of those to which the present invention pertains.

Applicants assert that it is well known to those skilled in the art that several enzymes and inhibitors can be expressed in tissues of transgenic animals without harmful effects. One of the first proteins to be expressed in the mammary gland of transgenic animals using the mWAP promoter was tissue plasminogen activator, which is important for the digestion of blood clots, by cleaving plasminogen into the active protease plasmin. (Gordon et al., 1987 Bio/Technology 5: 1183-1187; Pittius et al., Proc Natl Acad Sci, 1988, 85: 5874-5878). Urokinase, a plasminogen activator, that is normally produced in kidney and secreted into

urine was produced in transgenic mouse milk (Meade et al., 1990 Bio/Technology 8: 443-446). Urokinase cleaves plasminogen into the active protease, plasmin. The enzyme inhibitor human α1-antitrypsin, which prevents the enzyme elastase from causing tissue damage, was expressed in the milk of transgenic mice (Archibald et al., Proc Natl Acad Sci 87: 5178-5182, 1990), sheep (Ebert et al., Bio/Technology 9: 835-838, 1991) and in the blood of transgenic rabbits (Massoud et al., J. Biotechnol 18: 193-204, 1991). The production of bovine chymosin which digests milk proteins, in the mammary gland of 16 lines of transgenic rabbits is also known (Ernst et al., Dokl Akad Nauk 1995, 345: 555-558).

The Examiner is also of the opinion that the specification does not teach any 3' urinary tract-specific regulatory sequence that functions to target protein expression to the urinary tract or that is specific to all urinary tract tissue. Applicants respectfully direct the Examiner to Example 3, page 42 of the specification, which teaches examples of "3' regulatory sequences, including a polyadenylation sequence, that results in the expression of said DNA sequences in the urinary tract cells." Such examples include, but are not limited to, 3' regulatory sequences derived from uromodulin, uroplakin,renin, erythropoietin, apolipoprotein e, aquaporin, nephrocalcin, osteopontin-k / uropontin, urinary kallikrein and urinary thrombomodulin genes, as described.

Applicant reiterate that operably linking regulatory sequences to a DNA sequence that encodes a protein or peptide that degrades or detoxifies organic material and providing a transgenic animal that has stably integrated this DNA sequence is well within the skilled of the artisan using the specification, sequences and knowledge in the transgenic field. For all of the reasons set forth above, it is requested that this rejection be withdrawn.

3. 35 U.S.C. § 102

Claims 45-49, 51, 52, 54-59, 61, 62, 64 and 65

Claims 45-49, 51, 52, 54-59, 61, 62, 64 and 65 are rejected as anticipated by Sympson *et al.*("Sympson"). The Examiner alleges that Sympson teaches the production of a transgenic mouse expressing rat stromelysin-1 cDNA under the control of the WAP promoter. The Examiner considers that the WAP promoter directs expression inherently into the kidney and results in protein production in the urine. Sympson teaches the use of stromelysin-1 to

degrade extracellular matrix proteins, such as laminin and fibronectin. Sympson does not teach the use of stromelysin-1 present in urine to degrade waste proteins. The purpose of Sympson's study was to examine the effect of *inappropriate* expression of stromelysin-1 in mammary glands of pregnant female mice. Although applicants believe that Sympson does not inherently disclose the claimed method and transgenic animal, applicants have amended claims 45 and 56 to recite that the "organic material" is feces, urine, a microbe, a chemical pollutant or a by-product thereof, and a food product or a by-product thereof as supported in the specification on page 17, line 33 to page 18, line 2 and as in original claims 6 and 11. It is requested that this rejection be withdrawn.

4. 35 U.S.C. § 103

Claims 45-47, 51, 54, 55 and 67-69

Claims 45-47, 51, 54, 55 and 67-69 are rejected as anticipated by Sympson *et al.*("Sympson") in view of Wen *et al.* ("Wen") and Lubon *et al* (U.S. 5,880,327) The Examiner alleges that Sympson teaches the production of a transgenic mouse expressing rat stromelysin-1 cDNA under control of the WAP promoter and that WAP promoter directs expressin inherently in the kidney resulting in protein production in the urine. The Examiner admits that Sympson does not suggest that stromelysin-1 gene could be isolated from the urine but applies Wen and Lubon as teaching that proteins of interest could be isolated from other tissues, such as urine, using the WAP promoter. The Examiner concludes that it would have been obvious to collect and isolate stromelysin-1 protein from the urine of the transgenic mice produced by Sympson with a reasonable expectation of success.

The arguments made above against the anticipation rejection based on Sympson applies to this rejection in regard to why Sympson does not disclose the claimed invention. Moreover, the secondary references do not cure the deficiencies in this rejection. Further the claims clearly define the type of organic material that is degraded. It is requested that this rejection be withdrawn.

Conclusion

In light of the foregoing amendments and remarks, applicants submit that all claims are in condition for allowance, and they solicit an early indication to that effect. Should the Examiner believe that further discussion of any remaining issues would advance the prosecution, he is invited to contact the undersigned at the telephone number listed below.

Respectfully submitted,

March 19, 2001
Date

Jayme A. Huleatt
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MARKED-UP CLAIMS:

45. (Twice amended) A method of producing in the urine of a non-human transgenic animal a protein or a peptide that degrades or detoxifies organic material, said method comprising:

(a) providing a non-human transgenic animal having stably integrated into its genome an exogenous gene encoding a protein or peptide that degrades or detoxifies organic material comprising expression regulatory sequencesoperably linked to said exogenous gene encoding said protein or peptide, wherein said organic material is feces, urine, microbe, a chemical pollutant or a by-product thereof, a food product or a by-product thereof; and

(b) allowing said exogenous gene encoding said protein or peptide to be expressed and said protein or peptide to be secreted into the urine of said transgenic animal.

56. (Twice Amended) A non-human transgenic animal that produces in its urine a protein or peptide that degrades or detoxifies organic material, wherein said transgenic animal has stably integrated into its genome an exogenous gene encoding a protein or peptide that degrades or detoxifies organic material comprising expression regulatory sequencesoperably linked to said exogenous gene encoding said protein or peptide that is detectable in the urine of said transgenic animal, wherein said organic material is feces, urine, microbe, a chemical pollutant or a by-product thereof, a food product or a by-product thereof.

63. (Twice Amended) The transgenic animal of claim ~~56~~ 72, wherein said kidney-specific regulatory sequence comprises a uromodulin promoter.

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Genetics

Attach #22
08/982,284

High-level expression of biologically active human α_1-antitrypsin in the milk of transgenic mice

(emphysema/elastase/antiprotease/recombinant DNA/therapeutic proteins)

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Communicated by Mary F. Lyon, March 26, 1990 (received for review February 16, 1990)

ABSTRACT **Reduced circulating levels of α_1-antitrypsin (α_1AT) are associated with certain α_1AT genotypes and increased susceptibility to emphysema. Unfortunately, the amounts of α_1AT that would be required for replacement therapy are beyond the capacity of plasma fractionation and mammalian cell culture systems. Thus, we have examined the potential of transgenic animals as an alternative means of producing human α_1AT. A hybrid gene constructed by using sequences from the ovine milk protein gene β-lactoglobulin fused to an α_1AT "minigene" was used to generate transgenic mice. Of 13 independent transgenic mice and mouse lines, 5 expressed the hybrid gene in the mammary gland, 5 in the salivary glands, and 2 in both these tissues. Human α_1AT was secreted into the milk of each of the 7 mice and mouse lines that expressed the hybrid gene in the mammary gland. Four of these mammary-expressing transgenic mice and mouse lines produced concentrations of at least 0.5 mg of α_1AT per ml in their milk; one line (AATB 35) produced 7 mg of this protein per ml. α_1AT from transgenic mouse milk was similar in size to human plasma-derived α_1AT and showed a similar capacity to inhibit trypsin. Expression at equivalent levels in transgenic sheep or cattle would yield sufficient α_1AT for therapeutic purposes.**

Genetic deficiencies of α_1-antitrypsin (α_1AT) in humans are common and result in an increased susceptibility to emphysema (1). Human α_1AT is a 394-amino acid glycoprotein that acts as a suicide inhibitor of a wide range of serine proteases. In humans, the α_1AT gene is expressed in a variety of tissues, including macrophages, kidney, small intestine, pancreas, and liver; the latter is the primary site of expression (1, 2). In normal humans, more than 2 g of α_1AT is synthesized daily, resulting in a serum concentration of ≈2 mg/ml.

The primary function of α_1AT is to inhibit neutrophil elastase and thus prevent this protease from causing excessive tissue damage (1). The S and Z α_1AT alleles are relatively common (≈0.03 and 0.02, respectively) and encode proteins that have reduced stability (S) or are poorly secreted (Z), although they exhibit normal antiprotease activity. Individuals with the SZ and ZZ genotypes have significantly reduced concentrations of α_1AT (<0.8 mg/ml) and are at risk of developing the degenerative lung disease emphysema, particularly if they smoke.

Since α_1AT normally circulates at 2 mg/ml and has a half-life of 6 days, considerable quantities (≈4 g per week per patient) would be required for replacement therapy for afflicted individuals (3), which amounts to 4000–8000 kg annually to treat the ZZ homozygote population of the United States (4). Such large amounts of protein will be available only if recombinant DNA technology is used for production. However, although α_1AT does not require its carbohydrate side chains for activity, the *in vivo* half-life of nonglycosylated α_1AT (expressed in yeast) is 50-fold lower than that of plasma-derived α_1AT (4). Therefore it would seem prudent to produce α_1AT in a mammalian expression system capable of making the appropriate posttranslational modifications. Unfortunately, large-scale culture of mammalian cells is expensive and technically demanding and thus far has failed to match the yields necessary for high dosage therapeutics, as exemplified by α_1AT.

As an alternative to genetically engineered cell lines, Palmiter *et al.* (5) proposed that valuable proteins could be harvested from transgenic animals. We have argued that the mammary gland is the organ of choice for the expression of recombinant proteins (6, 7) because large amounts of protein can be synthesized by the mammary gland, secreted into milk, and collected easily without detriment to the animal. We have decided to use sheep for this purpose and have recently demonstrated the production of human factor IX and α_1AT in the milk of transgenic sheep (8, 9). In these sheep, and also in transgenic mice carrying the same hybrid genes, the levels of expression of the transgenes were low. The comparisons of the performance of these hybrid genes (FIXA and AATA) in transgenic sheep and mice are the subject of separate studies (M.M., A.L.A., S. Harris, J.P.S., B. Whitelaw, I. Wilmut, and A.J.C., unpublished results; M.M., H. Bessos, C. Prowse, J.P.S., B. Whitelaw, I. Wilmut, and A.J.C., unpublished results).

As money and time preclude the use of large animals to test and refine DNA constructs for efficient expression, we have elected to carry out these experiments in transgenic mice. We previously showed that the gene encoding a sheep milk protein, β-lactoglobulin (BLG), was expressed efficiently and abundantly in the mammary gland of transgenic mice (10). Here we show that sequences derived from this gene can be used to direct expression of human α_1AT sequences in the mammary gland, yielding high levels of human α_1AT in milk.

METHODS

Hybrid Gene Construction and Production of Transgenic Mice. A hybrid gene (referred to as AATB, see Fig. 1) was elaborated in which the *Pvu* II site within the 5′ untranslated sequences of the ovine BLG clone SS1 (11, 12) was fused to the *Taq* I site in the 5′ untranslated sequences of α_1AT. The first α_1AT intron was excluded by using DNA sequences from a cDNA clone, p8α1ppg, which encodes the M_1 variant of α_1AT (13), as the source of the first 80 base pairs of α_1AT sequences, extending up to the *Bam*HI site in the second exon. The remainder of the α_1AT "minigene" comprises a

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Abbreviations: α_1AT, α_1-antitrypsin; BLG, β-lactoglobulin; G_n, generation n; RID, radial immunodiffusion.

†To whom reprint requests should be addressed.

6.5-kilobase (kb) *Bam*HI fragment from the human α_1AT genomic clone pATp7 [also encoding the M_1 variant of α_1AT (14)]. The construct was elaborated in the vector pPOLYIII-I (15), enabling excision of the 10.6-kb insert by using *Not* I sites in the polylinker sequences. Gel-purified insert DNA was microinjected into pronuclear mouse eggs [collected from (C57BL/6 × CBA)F_1 mice after mating with F_1 stud males] in order to generate transgenic mice (10, 16). Lines were propogated by mating with F_1 mice.

DNA and RNA Analysis. DNA (for Southern blot analysis) prepared from tail biopsies was digested with restriction enzyme(s), subjected to agarose gel electrophoresis, blotted to Hybond N (Amersham) nylon membranes, and probed with ^{32}P-labeled AATB DNA sequences. RNA was prepared from lactating mice 11 days after parturition by standard methods (17, 18). Aliquots (10 μg) of total RNA were fractionated on denaturing Mops/formaldehyde (1–1.5%) agarose gels, transferred to Hybond N membranes (Amersham), and probed with a ^{32}P-labeled 243-base-pair *Taq* I–*Pst* I fragment derived from the 3′ end of p8α1ppg (13), which allows mouse and human α_1AT mRNAs to be distinguished. DNA probes were labeled by using random primers (19), and hybridizations were carried out as described by Church and Gilbert (20).

Analysis of Milk. Milk was collected from lactating females 11 days after parturition as described by Simons *et al.* (10). Mouse milk was diluted 1:5 in distilled water, and fat was removed after centrifugation. To prepare whey, 1.0 M HCl was added to give a final pH of 4.5, to precipitate the caseins, which were then removed by centrifugation.

Diluted milk or whey samples were solubilized by boiling in loading buffer prior to discontinuous SDS/polyacrylamide (8% or 10%) gel electrophoresis (21) and immunoblotting analysis (22). Human α_1AT was identified on immunoblot filters by using goat anti-α_1AT serum [Protein Reference Unit (PRU), Royal Hallamshire Hospital, Sheffield S10 2JF] and anti-sheep/goat IgG serum conjugated to horseradish peroxidase [Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke, Lanarkshire, ML8 5ES]. Amounts of human α_1AT in mouse milk were measured by radial immunodiffusion (RID) and radioimmunoassay (RIA). RID estimates were obtained by using LC-Partigen RID plates (Behring Diagnostics) according to the manufacturer's instructions. RIAs were performed according to standard procedures (23) with goat anti-human α_1AT antiserum (PRU) and donkey anti-goat IgG (SAPU). Human α_1AT, purified from plasma by using a modification of the method described by Laurell *et al.* (24), was iodinated by using chloramine T and used as the tracer, and pooled human plasma was employed for calibration. The detection limits of these methods were 40 μg/ml (RID) and 5 μg/ml (RIA), respectively, when applied to defatted murine milk samples, and results were validated by using known amounts of human plasma/serum added to control mouse milk.

Trypsin Inhibitory Activity. Dilutions of defatted milk or plasma (40 μl) were incubated at room temperature with equal volumes of trypsin (bovine pancreatic type III, Sigma) at 200 units/ml in 0.1 M Tris/0.15 M NaCl/3 mM sodium azide, pH 8.0 for 5 min, before addition of 40 μl of chromogenic substrate S-2222 (KabiVitrum) (25). After 2.5 min, acetic acid was added to a final concentration of 8.5% to stop the reaction, and absorbances at 405 nm were read immediately.

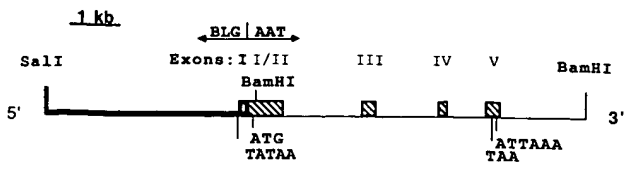

FIG. 1. The AATB construct comprises ≈4.0 kb of the 5′ end of the ovine BLG clone SS1 (11, 12) fused to a minigene encoding human α_1AT. Thick line, 5′ BLG sequences; open box, BLG exon 1 sequences; hatched boxes, α_1AT exons; thin lines, α_1AT introns and 3′ flanking regions. The position of the BLG TATA box and also the α_1AT initiation codon, stop codon, and polyadenylylation site are shown.

RESULTS

Generation of Transgenic Mice. To direct expression of α_1AT to the mammary gland of transgenic mice, a hybrid gene (AATB) was elaborated, comprising, ≈4.0 kb of the 5′ end of the ovine BLG gene fused to a minigene encoding human α_1AT (Fig. 1). The hybrid gene (AATB) was microinjected into pronuclei of fertilized mouse eggs (n = 993). Analysis of DNA prepared from tail biopsies showed that 21 of the 122 generation zero (G_0) animals carried the AATB construct.

Expression of the AATB Transgene. Expression of the transgene was assessed by analyzing RNA and milk from lactating females that were generally either G_0 animals or the transgenic G_1 offspring of G_0 males. Three patterns of human α_1AT RNA expression were observed after Northern blot analysis (Fig. 2). In some animals and lines, expression was limited to the mammary gland, whereas in others it was confined to the salivary gland. There were two lines where transcripts were seen in both the mammary and the salivary glands (Table 1). As judged by comparison with human liver RNA and HepG2 RNA, both mammary and salivary transcripts were, as expected, the same size as human liver α_1AT mRNA. One line in particular, AATB 35, showed extremely high levels of expression of α_1AT mRNA in the mammary gland, comparable to the level observed in human liver.

Production of Human α_1AT in Milk. Milk was analyzed by SDS/PAGE and immunoblotting for the presence of human α_1AT protein (Fig. 3*a*). Human α_1AT was present in milk from all the transgenic animals that had detectable levels of human α_1AT mRNA in the mammary gland but was not detected in those that did not express the transgene or expressed it only in the salivary gland. The antiserum to human α_1AT cross-reacted with an endogenous mouse protein present in milk, probably murine α_1AT. The most

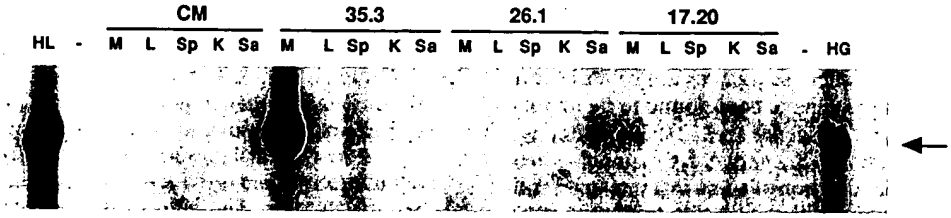

FIG. 2. Northern blot analysis of total RNA from transgenic mice (numbers AATB 35.3, AATB 26.1, and AATB 17.20) and a control C57BL/6 mouse (CM). The tissues analyzed were mammary (M), liver (L), spleen (Sp), kidney (K), and salivary (Sa). Control lanes: HL, human liver RNA; HG, HepG2 RNA (26). The ≈1400-nucleotide α_1AT transcripts are indicated by an arrow. Ten micrograms of total RNA was loaded except for HL, 35.3 M, and HG, which contain 1 μg of sample RNA with 9 μg of control mouse mammary RNA.

Table 1. Summary of the pattern of expression of AATB RNA in transgenic mice

Animal/line	Sex*	Copy no.	Mammary	Salivary
15	M	3	+†	−
17	M	10	+	+
26	M	20	−	+
28	M	3	−	−
35	M	1	+	+
44	F	15	+	−
45	F	2	+	−
65	F	3	+	−
69	F	2	+	−
78	M	10	−	+
79	M	15	−	+
105	F	20	−	+
107	F	15	−	+

Expression was analyzed by Northern blot analysis of tissues from lactating G_0 females or, where the founder was male, from G_1 females that had inherited the transgene. No human α_1AT RNA was detected in liver, spleen, kidney, or heart. Copy numbers were estimated by Southern blotting relative to copy number controls.
*Sex of the G_0 animals.
†α_1AT transcripts were detected only in poly(A)$^+$ RNA in one of two animals analyzed.

prominent human α_1AT bands in transgenic mouse milk had electrophoretic mobilities similar, but not identical, to the major bands observed in samples of purified human α_1AT or pooled human plasma.

The concentrations of human α_1AT in transgenic mouse milk were measured by RID and RIA (Table 2). The results obtained with the two methods of measurement were similar. Concentrations ranged from 6 μg/ml (mouse 15.20) to more than 7 mg/ml (mouse 35.3). Of the seven animals and lines that expressed the transgene in the mammary gland, four yielded concentrations of α_1AT of at least 0.5 mg/ml.

Milk from Transgenic Mice Has Enhanced Trypsin-Inhibitory Activity. Milk samples from line AATB 35 were shown to have high levels of trypsin-inhibitory activity when compared with milk from nontransgenic mice (Fig. 4*a*). When milk from line AATB 35 mice was compared with human plasma, it was evident that equivalent amounts of plasma and milk α_1AT had similar biological activities (Fig. 4*b*). Milk from line AATB 17 mice was also shown to have greater levels of antitrypsin activity than milk from control mice. The trypsin-inhibitory capacities of milk from lines 17 and 35 were in accord with expectations based on the α_1AT contents of these milks as measured by immunological methods.

DISCUSSION

For the reasons outlined above, we sought to harness the high protein synthetic capacity of the mammary gland of transgenic animals as a source of recombinant α_1AT. To this end, we elaborated a hybrid gene (AATB) by fusing the promoter and 5′ flanking sequences from the abundantly expressed ovine milk protein BLG to a human α_1AT minigene. The construction of such hybrid genes and their excision from vectors is eased if the component sequences are kept as short as possible. However, introns have been found to be important for the expression of transgenes (ref. 27; B. Whitelaw, M.M., A.L.A., S. Harris, J.P.S., and A.J.C., unpublished results). Nevertheless, the deletion of some intron(s) may still allow high-level expression while facilitating transgene construction. The omission of the first α_1AT (5.3-kb) intron made the elaboration of the construct simpler and excluded a 429-base-pair open reading frame, an *Alu* repeat, and a pseudo transcription initiation sequence (28).

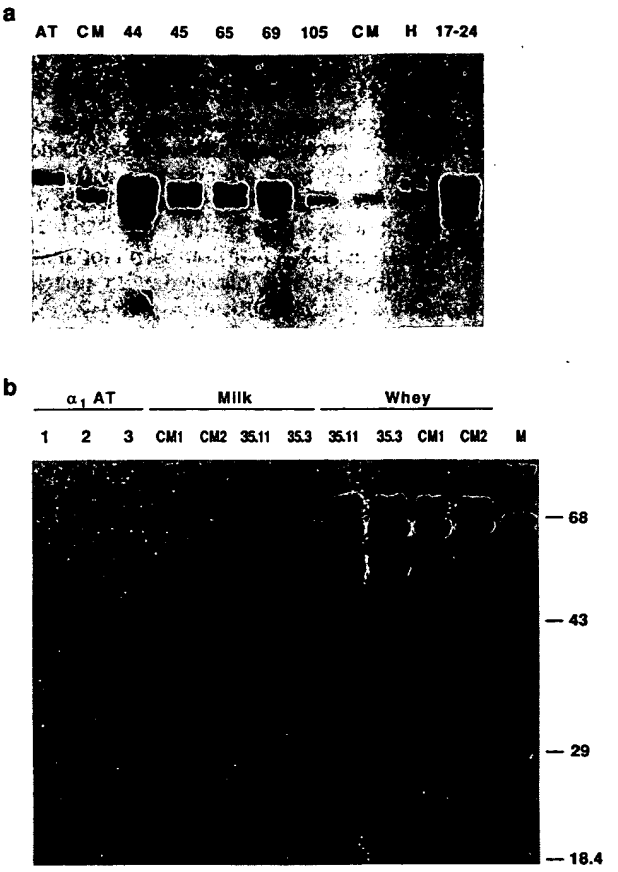

FIG. 3. Electrophoretic analysis of milk proteins. (*a*) Immunoblot. Wheys, equivalent to 1.5 μl of milk, from transgenic mice (numbered lanes) and control mice (CM), 0.25 μg of purified human α_1AT (AT) (Sigma), and 0.05 μl of pooled human sera (H) were immunoblotted and probed for human α_1AT. (*b*) SDS/PAGE gel. Defatted milk and whey samples from control mice (CM) and two transgenic G_1 females from line 35 (numbered lanes) were electrophoresed alongside dilutions of purified human α_1AT (Sigma; lane 1, 5 μg; lane 2, 2.5 μg; lane 3, 1 μg) and molecular weight markers (M) (GIBCO, BRL) and stained with Coomassie blue.

The finding of mammary gland expression of the AATB construct in seven transgenic individuals and lines confirmed the efficacy of the construct design. However, salivary expression using the BLG promoter was not anticipated. We

Table 2. Measurements of human α_1AT present in transgenic mouse milk as determined by immunoblotting (Blot), RID, and RIA

Animal/line	Generation	Blot	RID, μg/ml (*n*)	RIA, μg/ml
15.10	G_1	+	— (1)	6
15.20	G_1	−	— (1)	—
17.23	G_1	+	463 (5)	ND
17.24	G_1	+	556 (6)	520
17.5.1	G_2	+	990 (2)	1055
17.5.4	G_2	+	407 (2)	390
17.5.9	G_2	+	606 (3)	490
17.5.16	G_2	+	730 (2)	680
35.3	G_1	+	7738 (2)	9000
35.11	G_1	+	6215 (2)	5700
44	G_0	+	879 (2)	920
45	G_0	+	84 (2)	59
65	G_0	+	83 (2)	46
69	G_0	+	695 (2)	445

n, Number of assays performed; ND, not determined.

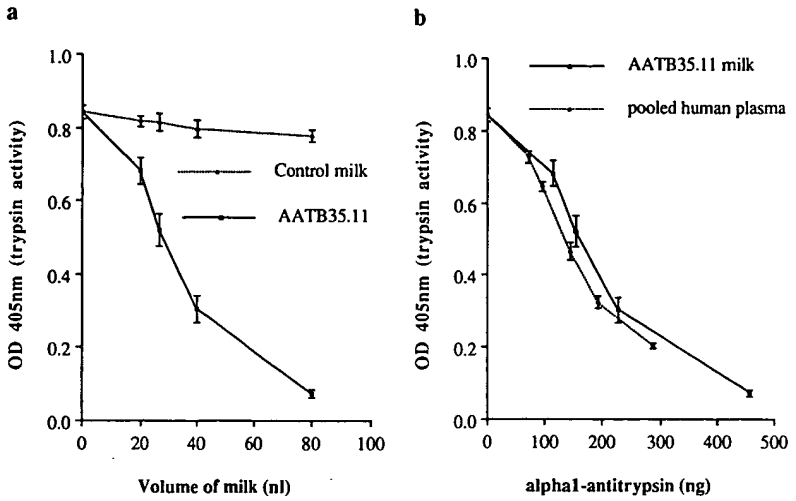

FIG. 4. Trypsin-inhibitory activity of transgenic mouse milk. (*a*) A comparison of the trypsin-inhibitory capacities of milk from transgenic mouse AATB 35.11 and pooled milk from nontransgenic mice. (*b*) A comparison of the trypsin-inhibitory capacities of the human α_1AT in milk from mouse AATB 35.11 and in pooled human plasma; the human α_1AT contents of the diluted milk and plasma samples were calculated from RID estimates of the concentrations of the corresponding milk and plasma.

eliminated mammary gland contamination of salivary gland RNA as the cause of this result by reprobing with mammary-specific probes (data not shown). We have not detected significant salivary expression of a variety of other transgenes comprising the BLG gene or its hybrid derivatives (ref. 10; A.J.C., A.L.A., S. Harris, M.M., J.P.S., and B. Whitelaw, unpublished observations). All these other transgenes share 3′ BLG sequences that are absent from the AATB construct. Interestingly, transgenic mice carrying the human α_1AT gene show expression in the salivary gland (29). Several other groups have also reported salivary gland expression of hybrid genes in which the promoter and 5′ flanking sequences were also derived from mammary-specific genes (30–33). Thus, it is possible that the downstream BLG sequences contain a salivary gland-specific negative regulatory element or that positive regulatory elements within the α_1AT sequences direct expression to this tissue.

Although the electrophoretic mobilities, in SDS/PAGE, of α_1AT from transgenic mouse milk or human plasma are similar, the electrophoretic pattern of α_1AT proteins observed in transgenic mouse milk appears more complex. This may reflect differences in the posttranslational modifications of the proteins produced in human liver and mouse mammary gland. Alternatively, human α_1AT produced in mouse milk may be more susceptible to degradation during secretion or storage.

Within lines of transgenic mice, some variation in expression was observed. In line 15, low-level α_1AT expression was detected in only one of the two animals analyzed, and in line 17, in which both G_1 and G_2 animals were analyzed, an $\approx$2-fold variation in α_1AT concentration was observed. This may simply reflect a variation in the total protein content of individual milk samples (10) or variation in the level of transgene expression within a line due to nonuniform genetic backgrounds. We have also noted considerable variation in the level of transgene expression within a line of mice carrying the BLG gene (M.M., unpublished observations).

The level of human α_1AT in the milk of line 35 mice is very high, as expected from the level of α_1AT mRNA observed in the mammary gland. The α_1AT is clearly evident on Coomassie blue-stained gels of total milk proteins (Fig. 3*b*). Densitometry of stained gels showed that human α_1AT comprises $\approx$10% of total milk proteins and more than 30% of the whey proteins. These proportions compare favorably with those obtained for expression of α_1AT in bacteria (15% of total cell protein) and yeast (3% of total soluble proteins) and particularly so when compared with eukaryotic cell culture expression (<1 μg per 10^6 cells per 24 hr) (34–37).

The human α_1AT present in the milk of transgenic mice from lines 17 and 35 was shown to be biologically active in a trypsin-inhibition assay. When transgenic mouse milk (mouse 35.11) and pooled human plasma were compared, it was evident that equivalent amounts of plasma and recombinant α_1AT had similar capacities to inhibit trypsin (Fig. 4*b*), indicating that α_1AT synthesized in the mammary gland and secreted into milk is as biologically active as that derived from plasma.

In December 1987, the U.S. Food and Drug Administration licensed the use of α_1AT in replacement therapy (1). The methods by which α_1AT might be delivered to the critical lung locations include intravenous infusions, aerosol sprays, and gene therapy (38, 39). It seems likely that gene therapy will be very expensive and not readily available to the many individuals suffering from α_1AT deficiency. For replacement therapy by means of aerosols or intravenous infusions to be generally available, large quantities of biologically active and correctly glycosylated α_1AT will be required.

In this paper we have described the production of transgenic mice expressing high levels of biologically active human α_1AT in their milk. The levels of expression in line 35 are of particular interest and, to our knowledge, represent one of the highest levels of expression of a recombinant protein in any mammalian expression system, including transgenic mice and sheep (9, 40, 41). High-milk-yielding breeds of sheep, such as the East Friesland, can produce up to 400 liters per lactation. Therefore transgenic sheep that express α_1AT at the levels observed in line 35 could produce up to 3 kg of α_1AT at each lactation, a level of production that might be capable of supplying the large quantities required for replacement therapy.

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Biochemistry

A milk protein gene promoter directs the expression of human tissue plasminogen activator cDNA to the mammary gland in transgenic mice

(whey acidic protein/mouse mammary gland/lactation/hormone regulation/tissue-specific expression)

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ABSTRACT **Whey acidic protein (WAP) is a major whey protein in mouse milk. Its gene is expressed in the lactating mammary gland and is inducible by steroid and peptide h rmones. A series of transgenic mice containing a hybrid gene in which human tissue plasminogen activator (tPA) cDNA is under the control of the murine WAP gene promoter had previously been generated. In this study, 21 tissues from lactating and virgin transgenic female mice containing the WAP-tPA hybrid gene were screened for the distribution of murine WAP and human tPA transcripts. Like the endogenous WAP RNA, WAP-tPA RNA was expressed predominantly in mammary gland tissue and appeared to be inducible by lactation. Whereas WAP transcripts were not detected in 22 tissues of virgin mice, low levels of WAP-tPA RNA, which were not modulated during lactation, were found in tongue, kidney, and sublingual gland. These studies demonstrate that the WAP gene promoter can target the expression of a transgene to the mammary gland and that this expression is inducible during lactation.**

Synthesis and secretion of many milk proteins is specific to the lactating mammary gland and expression of the corresponding genes is modulated by steroid and peptide hormones (1). Our goal is to identify and characterize control elements governing milk protein gene expression. Although cell culture systems have been used to define DNA-regulatory elements such as promoters, enhancers, and hormone-responsive elements in a number of genes, hormone-regulated and tissue-specific expression of milk protein genes has not yet been demonstrated in cell lines. The transgenic mouse appears to be an appropriate system for the characterization of gene-regulatory elements within the context of the organism (2–8).

The whey acidic protein (WAP) gene, encoding a major murine milk protein (9), has been cloned (10, 11) and putative regulatory elements in the promoter region have been identified (12). We have previously described the generation of transgenic mice carrying hybrid genes containing the murine WAP gene promoter and the human Ha-*ras* gene (13) or human tissue plasminogen activator (tPA) (14) cDNA. Human tPA is a protease capable of fibrinolysis and has been shown to have efficacy in the treatment of clotting disorders. Aside from providing information about WAP gene regulation, the transgenic mice carrying the WAP-tPA hybrid gene represent a model system for the cost-effective production of human therapeutic proteins in milk.

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In this report, we identify transgenic mice carrying the WAP-tPA hybrid gene, analyze the authenticity of tPA product secreted into milk, and compare the steady-state levels of WAP and WAP-tPA RNAs in several tissues from lactating and virgin transgenic animals.

MATERIALS AND METHODS

Production of Transgenic Mice. The hybrid gene WAP-tPA, consisting of the promoter region of the WAP gene (−2600 to +24) fused to the cDNA coding for human tPA, was introduced into mouse embryos as a *Hin*dIII–*Bam*HI fragment (14). DNA was microinjected into one-cell fertilized eggs and transgenic mice were derived as described (14).

Isolation of DNA and RNA. A short segment of mouse tails was removed and genomic DNA was prepared by the procedure of Hogan *et al.* (15). RNA was isolated by extraction with acid guanidinium isothiocyanate/phenol/chloroform (16). The integrity of RNA was confirmed on formaldehyde gels stained with ethidium bromide. Poly(A) RNA was prepared by chromatography on oligo(dT)-cellulose (Pharmacia; ref. 17).

DNA Analysis. Mice carrying the hybrid gene were identified by Southern blot analysis (18) of *Eco*RI-digested DNA extracted from tails using a probe specific for the promoter of the WAP gene. The probe consisted of a 1700-base-pair (bp) *Bgl* II–*Kpn* I fragment (−1700 to +24) of the WAP gene (11) labeled with ^{32}P by using random hexanucleotide primers (19).

Preparation of Antisense RNA Probes. A 641-bp *Hin*fI fragment from the WAP gene (−413 to +228, see ref. 11) was blunt ended with mung bean nuclease and cloned into the polylinker of the Bluescribe vector pBS (Stratagene, La Jolla, CA). After linearizing the plasmid at the *Xba* I site (nucleotide −88 in the WAP gene promoter), antisense RNA labeled with ^{32}P to a specific activity of 10^9 cpm/μg was produced with T7 RNA polymerase. For detection of WAP-tPA hybrid RNA, the probe was synthesized with T7 RNA polymerase from a plasmid containing the 220-bp *Xba* I–*Bgl* II fragment that spans the WAP-tPA junction (see Fig. 3).

RNase Analysis. Ten micrograms of total RNA isolated from tissue was incubated with 200,000 cpm of ^{32}P-labeled antisense RNA in 30 μl of 40 mM Pipes, pH 6.7/0.4 M NaCl/80% formamide as described (20). After denaturation at 85°C for 5 min, tubes were incubated at 37°C for 12 hr. RNase digestion was performed by adding 300 μl of 0.3 M NaCl/10 mM Tris·HCl, pH 7.5/5 mM EDTA/40 μg of RNase

Abbreviations: WAP, whey acidic protein; tPA, tissue plasminogen activator; MMTV, mouse mammary tumor virus; LTR, long terminal repeat.

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A per ml (Sigma)/10 μg of RNase T1 per ml followed by incubation for 15 min at 30°C. After phenol/chloroform extraction, the RNA was precipitated with ethanol and digested with 2 units of RNase H (Bethesda Research Laboratories) in 20 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/100 mM KCl for 20 min at 37°C. Following digestion with proteinase K for 15 min at 37°C, extraction with phenol/chloroform, and ethanol precipitation, RNA was dissolved in 80% formamide and analyzed by electrophoresis in a 6% polyacrylamide/8 M urea gel. The gels were dried on Whatman paper and subjected to autoradiography at −70°C with intensifying screens for 12–150 hr. We calculate that this assay was sufficiently sensitive to detect one molecule of WAP mRNA per cell.

tPA Assays. Assays were performed with an ELISA kit (Immunobind). The standard curves were performed in negative mouse milk diluted to a final concentration of 10% with phosphate-buffered saline, to which was added tPA supplied with the kit. All points of the control curve and the experimental assays have the background value (the value determined for negative mouse milk) subtracted. Values were confirmed by using a fibrin clot lysis assay (14) (data not shown).

Electrophoretic Transfer Blot. Samples of the whey fraction from milk, soluble material following acid precipitation of milk, along with purified Bowes melanoma tPA were run on a 10–20% $NaDodSO_4$/polyacrylamide gradient gel according to the method of Laemmli (21). The proteins were electrophoretically transferred to nitrocellulose and incubated with goat anti-human melanoma tPA (American Diagnostica, Greenwich, CT). The detection system involved a horseradish peroxidase-conjugated rabbit anti-goat IgG and was developed with 4-chloro-1-naphthol (Bio-Rad).

RESULTS

Expression of Human tPA in Mouse Milk. A hybrid gene consisting of 2.6 kilobases (kb) of upstream DNA from the WAP gene and the cDNA encoding human tPA (14) was introduced into mouse oocytes. The WAP portion of this fusion gene terminates in the 5′ untranslated region within the first exon of the WAP gene, 24 bp downstream of the transcriptional start site. The tPA part of the WAP-tPA hybrid gene contains the start site of translation and the native secretion signal sequence from tPA. Six transgenic mouse lines were identified containing the WAP-tPA hybrid gene. Some preliminary data on three of these lines have been presented (14).

The copy number of integrated WAP-tPA sequences varied between 3 and 50 in these lines (Fig. 1*A*). Tail DNA was digested with *Eco*RI, which cleaves 5′ of the WAP promoter and twice within the tPA gene. The DNA was separated in an agarose gel, transferred to a filter, and probed with 5′ flanking DNA of the WAP gene, thereby permitting detection of a WAP-tPA gene fragment and the endogenous WAP gene. The 1.7-kb WAP gene probe consistently hybridized to two *Eco*RI fragments. One is a 6.5-kb fragment indicative of the endogenous WAP gene, which served as an internal hybridization standard for a single-copy gene; the other is a 3.3-kb fragment from the WAP-tPA hybrid gene. Most of the hybrid genes appeared to be integrated as multiple head-to-tail copies and were inherited in Mendelian fashion (data not shown).

The WAP-tPA construction was designed to facilitate expression in lactating mammary epithelial cells and to allow secretion of the gene product, human tPA, into milk. Transgenic female mice from each line were mated in order to propagate the strains and to provide milk samples for analysis. Human tPA was immunologically detected in the milk of mice during midlactation in four of the six lines (Fig. 1*C*). In separate experiments, using an *in vitro* clot lysis assay (ref. 22; data not shown), the tPA from the milk of these transgenic animals was biologically active. To correlate the amount of tPA in the milk from the different lines with the expression of the hybrid gene, poly(A) RNA was prepared from lactating mammary glands and analyzed in an RNase protection assay. The amount of tPA in the milk of the different lines correlated with the relative concentrations of WAP-tPA hybrid RNA (Fig. 1 *B* and *C*). However, there was no direct correlation between the copy number of WAP-tPA genes and the level of tPA expression (Fig. 1). In fact, line 27 produced the highest amounts of human tPA in the milk but contained the smallest number of WAP-tPA gene copies. The amount of tPA found in milk of the line 27 was ≈50 μg/ml of milk as judged by an ELISA.

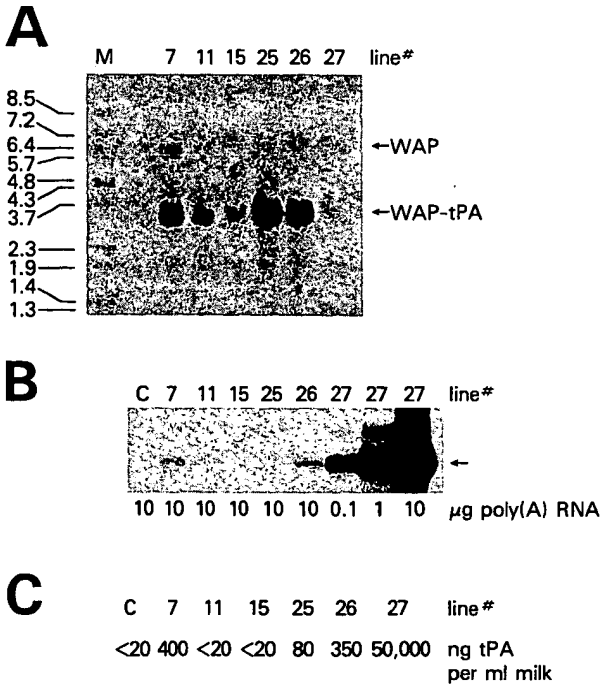

FIG. 1. (*A*) Southern blot analysis of genomic DNA from one member of each of the six transgenic lines. Tail DNA, 10 μg, from each animal was restricted with *Eco*RI and electrophoresed on a 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized with a probe of the WAP gene promoter. By using this probe the endogenous WAP gene (6.5-kb band) and a 3.3-kb fragment indicative of the WAP-tPA hybrid gene are visualized. The 3.3-kb fragment contains a 2.6-kb WAP gene promoter sequence and 700-bp tPA cDNA sequence. As can be estimated from the intensity of the 3.3-kb fragment, the copy number of the integrated transgene varied considerably between different lines. The size marker (lane M; sizes in kb) is a ^{32}P-labeled *Bst*EII digest of λ DNA. (*B*) RNase protection analysis of poly(A) RNA prepared from lactating mammary gland of mice from each of the six lines and nontransgenic control mice (lane C). The arrow indicates the WAP-tPA-specific transcript. The slightly larger transcript seen in line 27 might point to a second upstream start site used in a small percentage of the transcripts. (*C*) Average tPA concentration in the milk of lactating animals from the six lines and control mice (lane C), as determined independently by ELISA and clot lysis assay.

Fig. 2 shows an electrophoretic transfer blot characterizing the human tPA in milk of line 27 (Fig. 2, lane b). Several unique bands are evident in transgenic mouse milk as compared to the milk taken from a nontransgenic mouse (Fig. 2, lane a). For comparison, human tPA from Bowes melanoma (Fig. 2, lane c) consists of two predominant forms: single-chain material (64–65 kDa) and the more rapidly migrating two-chain form (30–36 kDa). The predominant band of the samples obtained from milk of line 27 after electrophoresis had a mobility similar to that of the two-chain

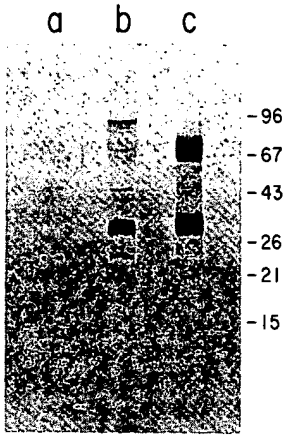

FIG. 2. Electrophoretic transfer blot of human tPA secreted into mouse milk. Milk samples were obtained from mice of line 27 (lane b) and from lactation-stage and age-matched nontransgenic control mice (lane a) by treatment of the mothers with oxytocin and manual manipulation as described (14). The whey fraction was prepared by removing the caseins with an acid treatment and centrifugation. The soluble (whey) fractions were neutralized and run on a polyacrylamide gel, and the human tPA samples were stained. Lane c, positive control from a Bowes melanoma cell line. Protein size markers are indicated (in kDa) on the right.

form of Bowes melanoma tPA (Fig. 2). Another protein band in milk of line 27 correlated well in size with the single-chain form, although the patterns are distorted by an excess of whey proteins in that molecular mass range (Fig. 2, lane b). The immunostained background pattern of the normal whey control may be due to crossreacting endogenous mouse tPA or nonspecific binding of whey proteins to the human polyclonal antibody. The presence of a higher molecular mass form of reactive protein than the single-chain form of tPA may represent a complex formed with a tPA inhibitor or an aggregate.

Tissue-Specific Expression of the WAP and WAP-tPA Gene. To assess tissue-specific expression of the endogenous WAP and WAP-tPA hybrid gene in line 27 we analyzed RNA from 21 tissues of lactating females by using an RNase protection assay. The WAP-specific template (Fig. 3*A*) consisted of a 322-bp fragment from nucleotide −88 to +234 within the first intron of the WAP gene. RNA synthesized *in vitro* from this template was hybridized with mammary gland RNA and digested with RNase. As predicted, a 112-bp fragment was generated, corresponding to the size of the first exon (Fig. 4*A*). Fig. 3*B* shows the corresponding WAP-tPA template. Upon hybridization of a probe derived from this template

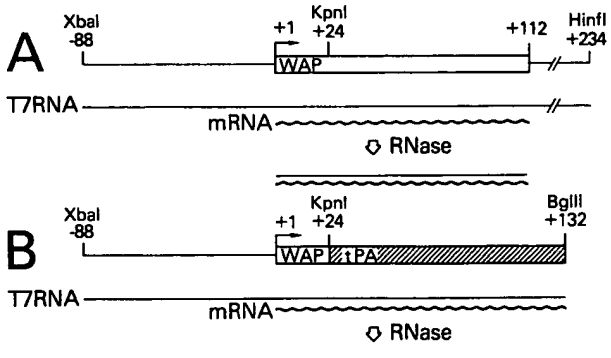

FIG. 3. Templates used for RNase mapping. The subcloned fragments used for antisense T7 polymerase transcription and generation of the WAP-specific RNA (*A*) and the WAP-tPA-specific RNA (*B*) are shown in the top portions. The predicted fragments that are protected from digestion with RNase are indicated by wavy lines.

with mammary gland RNA and RNase digestion, a 132-bp fragment indicative of the WAP-tPA hybrid transcript remained protected (Fig. 4*B*).

Steady-state levels of the two types of transcript were determined with the WAP- and WAP-tPA-specific probes in 21 tissues of lactating females of line 27. This line was chosen because it produced the highest levels of WAP-tPA RNA and human tPA protein. The major site of WAP and WAP-tPA RNA accumulation was the mammary gland (Fig. 4). The steady-state level of endogenous WAP RNA in the lactating mammary gland is ≈100-fold higher than that of WAP-tPA RNA. Lower levels of WAP RNA (by a factor of $\approx 10^4$–10^6) were detected in pituitary gland, pancreas, adrenal gland, tongue, liver, thymus, and heart atrium (Fig. 4*A*). WAP-tPA RNA at levels lower than in the lactating mammary gland (by a factor of at least 100) were detected in tongue, sublingual gland, and kidney (Fig. 4*B*). These results were confirmed by using sibling animals (data not shown).

Hormonal Regulation of WAP and WAP-tPA Gene Expression. Expression of the WAP gene in the mammary gland appears to be under the control of hormones (1). To assess whether control elements within the WAP-tPA hybrid gene were sufficient to confer hormone regulation, we analyzed 22 tissues of 6-week-old virgin females from line 27 for the presence of WAP and WAP-tPA RNA. No WAP-specific transcripts were detected in 10 μg of total RNA from each of the 22 tissues (Fig. 5*A*). In contrast, WAP-tPA RNA was found in the mammary gland, sublingual gland, tongue, and kidney of virgin females (Fig. 5*B*). Whereas the level of WAP-tPA RNA in the lactating mammary gland was about 100-fold higher than in virgin mammary glands, the levels of WAP-tPA RNA in kidney, sublingual gland, and tongue appeared to be unaffected by lactation. Since the relative number of mammary epithelial cells increases only about 5-fold during pregnancy (23), the 100-fold increase of WAP-tPA RNA is likely to reflect significant hormone regulation of the hybrid gene.

DISCUSSION

The WAP-tPA hybrid gene was stably integrated in six lines of transgenic mice. Active human tPA was found in milk obtained from mice of four lines and WAP-tPA hybrid RNA was found predominantly in the lactating mammary gland. These observations suggest that regulatory elements targeting expression to the lactating mammary gland are located within 2500 bp of WAP gene upstream sequence.

The level of active tPA in the milk of different lines varied over 3 orders of magnitude, with one line accumulating 50 μg/ml, or about 5% of the level of endogenous WAP. The varying amounts of human tPA in the milk correlated with the concentrations of WAP-tPA hybrid RNA in the lactating mammary glands of these mice, suggesting that differential secretion of tPA into the milk was not responsible for these differences. Also, no correlation could be established between the level of tPA expression and the copy number of WAP-tPA genes. This is in agreement with studies on several different genes (5). Rather, it appears likely that variability in the expression of the WAP-tPA gene may be due to position effects caused by the random insertion of the hybrid gene into the genome. This is corroborated by the variable expression pattern of another hybrid gene using the WAP gene promoter in transgenic mice, the WAP/Ha-*ras* oncogene construct (13).

Human tPA secreted into milk was verified to be authentic by electrophoretic transfer blot analysis using an antibody specific for human tPA. In contrast to the molecular form of the Bowes melanoma standard, recombinant tPA produced in mouse milk appeared to be primarily of the two-chain form. The predominance of a two-chain material could be due to endogenous plasmin present in milk. The material is biolog-

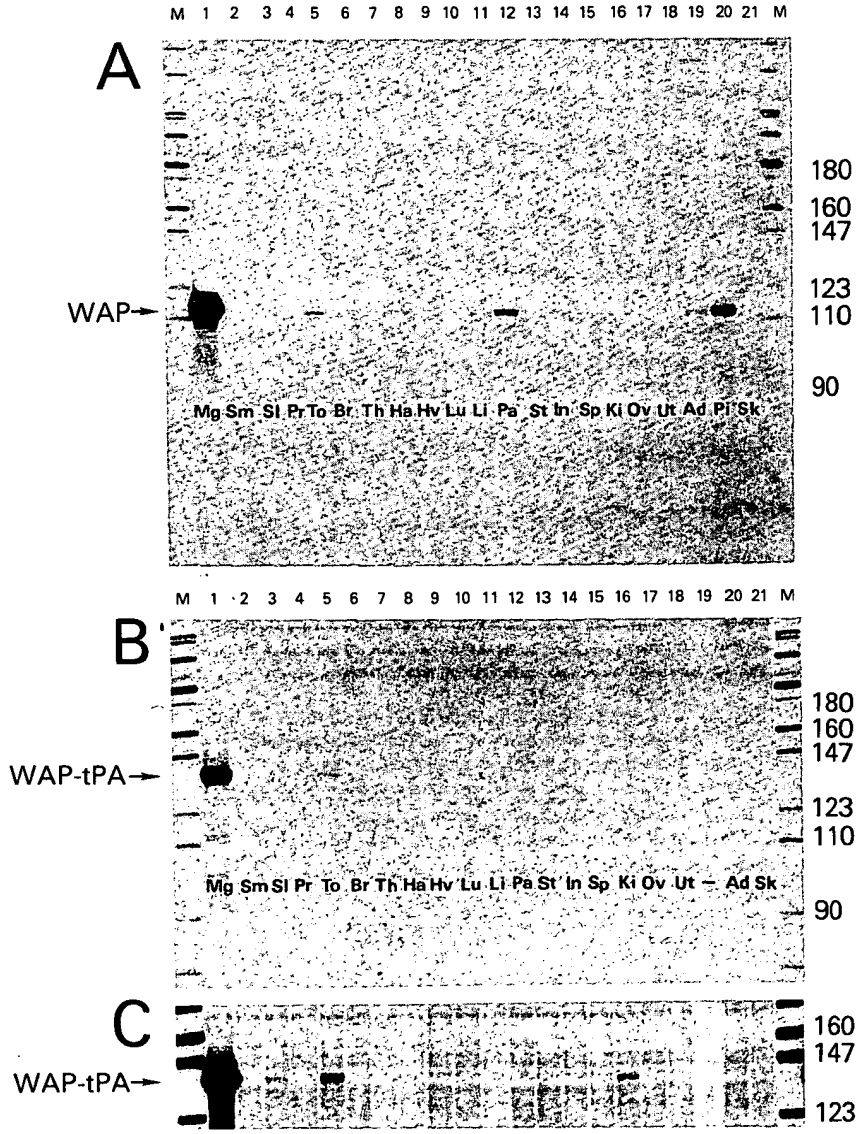

FIG. 4. Tissue distribution of endogenous WAP RNA (*A*) and WAP-tPA hybrid RNA (*B* and *C*) in a lactating mouse of line 27. RNA from 21 tissues was hybridized with radioactively labeled T7 RNA complementary to the WAP gene (*A*) or the WAP-tPA hybrid gene (*B* and *C*) and then digested with RNase; the reaction products were separated in a sequencing gel. The arrows indicate the protected 112-bp fragment encoding the first exon of endogenous WAP gene (*A*) and the 132-bp fragment indicative of the WAP-tPA hybrid RNA (*B* and *C*). The size marker (lanes M; sizes in bp) is a ^{32}P-labeled *Msp* I digest of pBR322. In lane 1 of *A*, 0.1 μg of total RNA from lactating mammary glands (Mg) was analyzed. All other lanes represent the hybridization signal upon incubation with 10 μg of total RNA. Sm, submaxillary gland; Sl, sublingual gland; Pr, parotid gland; To, tongue; Br, brain; Th, thymus; Ha, heart atrium; Hv, heart ventricle; Lu, lung; Li, liver; Pa, pancreas; St, stomach; In, intestine; Sp, spleen; Ki, kidney; Ov, ovary; Ut, uterus; Ad, adrenal gland; Pi, pituitary; Sk, skin. (*A* and *B*) Twelve-hour exposures. (*C*) Four-day exposure of *B*.

ically active, as judged by clot lysis assay; however, the exact specific activity of the human tPA produced in milk remains to be determined.

From our RNase protection studies it appears that the transcriptional start sites in the WAP-tPA and WAP genes are identical. Interestingly, in the WAP/Ha-*ras* transgenic mice a cryptic start site 3′ to the native one was utilized (13), suggesting that selection of transcriptional start sites may be influenced by sequences fused to the WAP gene promoter. Although the WAP-tPA gene is expressed predominantly in the lactating mammary gland, its expression pattern does not tightly follow that of the endogenous WAP gene; though no WAP RNA was found in any of 22 tissues from virgin females, low levels of WAP-tPA RNA were detected in mammary gland, sublingual gland, tongue, and kidney. The level of WAP-tPA transcripts in the mammary gland was greatly elevated during lactation, but the concentrations in sublingual gland, tongue, and kidney did not change significantly, suggesting that hormonal control of WAP-tPA gene expression is confined to the mammary gland. This notion is further supported by the presence of low amounts of WAP-tPA transcripts in sublingual gland, tongue, and kidney in transgenic males (data not shown).

The extent of hormonal induction and the absolute level of expression of the WAP-tPA gene are low compared to the endogenous WAP gene, suggesting that important cis-acting elements may be missing from the WAP gene promoter or may fail to function in this construct. Alternatively, the chromosomal environment and/or the sequence composition of the hybrid gene could affect the expression pattern as previously discussed in a different context (24). Complementary results were obtained with another well-studied mammary-specific expression system, the murine mammary tumor virus (MMTV). Endogenous MMTV sequences are transcribed preferentially in the mammary glands of lactating animals and critical control elements within the long terminal repeat (LTR) have been identified. However, studies with transgenic animals carrying the MMTV LTR fused to a variety of reporter genes revealed that the LTR can be expressed in at least 12 different organs (7, 8). In some cases, like spleen and salivary gland, transcription from the MMTV LTR was as high as in mammary tissue (7). Thus it appears that transcription of the endogenous WAP gene, and even more pronounced transcription of the MMTV LTR, in only lactating mammary epithelial cells must require other factors or sequences in addition to the promoter regions to achieve this higher order of specificity.

A detailed knowledge of mammary and hormone-regulated milk protein gene expression provides the basis for a biotechnology aimed at producing large amounts of complex proteins. Currently, macromolecular human pharmaceuticals—e.g., tPA and blood-clotting factors VIII and IX—are

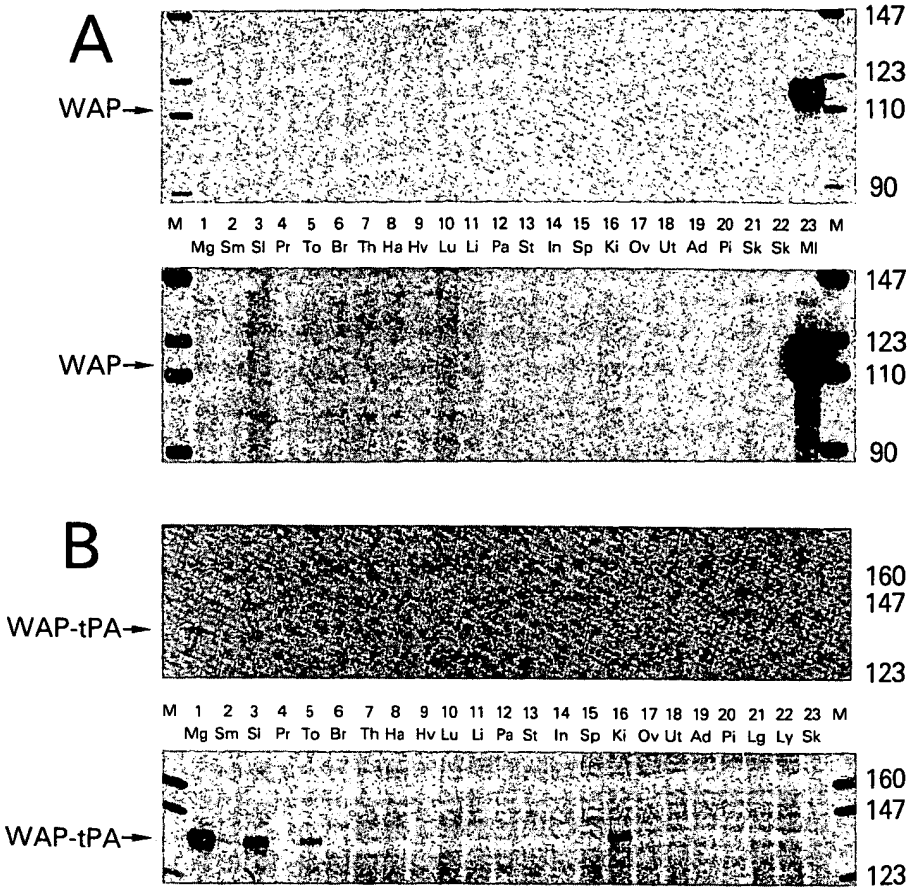

FIG. 5. Tissue distribution of WAP and WAP-tPA RNAs in a 6-week-old virgin female of line 27. Ten micrograms of total RNA from 22 tissues was analyzed for WAP and WAP-tPA RNA as described in the legend to Fig. 4. (*A*) Expression pattern of WAP RNA (12-hr and 4-day exposures). (*B*) Expression pattern of WAP-tPA hybrid RNA (24-hr and 5-day exposures). Lg, lacrimal gland; Ly, lymph node. See legend to Fig. 4 for other tissue abbreviations. In *A*, lane 23 (Ml), 0.1 μg of lactating mouse mammary gland RNA of line 27 was analyzed as a control. The size marker (lanes M; sizes in bp) is a ^{32}P-labeled *Msp* I digest of pBR322.

isolated from natural sources or are produced in tissue culture. Production of foreign proteins in the milk of transgenic dairy animals appears to be an alternative and cost-effective means for isolating human pharmaceuticals and other proteins in large quantities (25, 26). By using a mouse model system, we have succeeded in targeting the expression of active human tPA to the lactating mammary gland (ref. 14; this paper). Optimization of the mammary expression system and a transfer to dairy animals are dependent on further advances in our understanding of the molecular basis of regulated gene expression in the lactating mammary gland.

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PRODUCTION OF HUMAN TISSUE PLASMINOGEN ACTIVATOR IN TRANSGENIC MOUSE MILK

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We set out to express an exogenous gene in the mammary epithelium of transgenic mice in the hope that the encoded protein would be secreted into milk. The promoter and upstream regulatory sequences from the murine whey acid protein (WAP) gene were fused to cDNA encoding human tissue plasminogen activator (t-PA) with its endogenous secretion signal sequence. This hybrid gene was injected into mouse embryos, resultant transgenic mice were mated, and milk obtained from lactating females was shown to contain biologically active t-PA. This result establishes the feasibility of secretion into the milk of transgenic animals for production of biologically active heterologous proteins, and may provide a powerful method to produce such proteins on a large scale.

Genes injected into mouse embryos may be incorporated into the germ line and be expressed in patterns that mimic those of their endogenous counterparts[1,2]. The pattern of spatial and temporal expression of foreign genes in transgenic animals can be controlled by prior manipulation of the signals regulating gene expression. We introduced into mice a construct designed to express a foreign protein in the lactating mammary epithelium in which 5′ sequences from the whey acid protein gene were fused with a cDNA coding for tissue plasminogen activator. We demonstrate here that such an approach is a feasible means of expression of foreign proteins into secreted milk.

Whey acid protein (WAP) is the most abundant whey protein in mouse milk[3]. During lactation, the level of WAP RNA in the mammary gland increases approximately 340-fold from the barely detectable levels present in the mammary gland of virgin mice[4], and accumulates in lactating tissue at levels of about 15% of the total mRNA[4-6]. Expression of the WAP gene and the stabilization of its mRNA are subject to complex regulation by both steroid and peptide hormones[4], and putative regulatory protein binding sites within the WAP promoter have been described[7]. Since WAP is found in mouse milk at high levels and the gene had been previously cloned and characterized[8,9], we chose to utilize WAP upstream DNA as a promoter in our expression vector. By demonstrating secretion of a foreign protein into milk, the results reported here extend earlier observations showing upstream sequences from the WAP gene were able to target gene expression to the lactating mammary gland in transgenic mice[10].

RESULTS AND DISCUSSION

Construction of t-PA expression vector. A mammary expression vector was constructed in which 5′ sequences from the whey acid protein gene were fused with a cDNA coding for tissue plasminogen activator. t-PA has great potential clinical utility as an agent to dissolve fibrin clots and thus treat victims of myocardial infarction and other life threatening conditions. Its advantage relative to other pharmacological agents such as streptokinase and urokinase lies in its specificity for fibrin. Moreover, assay of its biological activity is both sensitive and convenient and an antibody kit is available for routine screening.

The t-PA gene utilized here was a cDNA clone from a human uterus cDNA library. The t-PA DNA sequence was determined previously and the protein expressed in C127 cells using bovine papilloma vectors[11]. The construct shown in Figure 1 (designated WAP-tPA) is a tripartite fusion consisting of 2.6 kb of upstream DNA from the WAP gene through the endogenous CAP site, t-PA cDNA beginning in the untranslated 5′ region, and the polyadenylation/termination signals from SV40. This tPA/SV40 polyadenylation cassette was characterized previously[11]. The secretion signal sequence in this construct derives from the native t-PA gene; the analogous signal encoding region from the WAP gene was removed in the construction. We did not know *a priori* whether the t-PA secretion signal would function efficiently in native mammary epithelial cells. However, since many proteins with different signal peptides are secreted efficiently by mammary cells and since milk proteins can be efficiently transported by membrane systems from other cells[12], it seemed likely that no specific signal sequence is required for secretion in mammary tissue.

Transient expression in tissue culture. To test WAP-tPA for its ability to specify production of secretable t-PA in mammary epithelial cells, the fusion gene was transfected into the mammary cell line, MCF7. Tissue culture supernatants collected 48 hours after transfection were loaded into wells of an assay plate as shown in Figure 2A. The assay consisted of lysis (clearing) of an artificial fibrin clot laid down as a matrix in agarose poured into the wells of a tissue culture plate. The degree of clearing, determined by estimating the diameter of the cleared ring

emanating from the sample loading well, indicates the amount of active t-PA in the sample. In five repetitions from two separate transfections, the level of t-PA secreted into the culture medium was found to be 2.5, 1.5, 10, 5, and 5 ng/ml. The same five samples were also assayed by ELISA using a polyclonal anti-human t-PA antibody. By this assay, the expression levels were either below the detection limit (approximately 2 ng/ml) or 11, 10, and 10 ng/ml, respectively. Thus, whether assayed by biological activity or immunologically, MCF7 cells transfected with WAP-tPA were able to secrete t-PA.

Generation of transgenic animals. The plasmid WAP-tPA was injected into one-cell pronuclear mouse embryos as a purified Hind-3/BamHI fragment containing no procaryotic sequences. The injected embryos were implanted into pseudopregnant females and 29 mice were born. Of these, seven were identified as being transgenic by diagnostic Southern blot hybridization with a human cDNA t-PA probe. Under conditions of high stringency, this probe does not hybridize with the endogenous mouse t-PA gene. The blot patterns of three positive mice, #wt1–26, wt1–25 and wt1–7 are shown in Figure 1. By comparison to the hybridization intensity obtained with positive controls, the number of copies of the injected fragment present in the genomes of these transgenic mice was estimated to be between 20 and 50. Digestion with SacI (lanes b–d) yielded a diagnostic band of 1.75 kb that spans the WAP and t-PA junction and hybridizes to the probe (Fig. 1). The intact plasmid digested with SacI was used as a positive control for this digest (lane a). Exogenous DNA injected into embryos tends to form concatomers even when introduced as a fragment with non-cohesive ends. The 2.3 kb band seen in lanes b–d corresponds to the 3′ end of the t-PA gene (which does not contain SacI sites), apparently ligated to the 5′ end of the WAP promoter, and through to the first Sac 1 site in the WAP DNA. The presence and size of this fragment is diagnostic of head-to-tail concatomers.

The EcoRI digest (control, lane h; experimentals, lane i–k) showed the expected 472 bp band internal to the t-PA gene. In addition, a 3.3 kb band can be seen that represents the 5′ region of the t-PA gene and extends through the WAP gene to 5′ boundary EcoRI site. Thus, despite the fact that the WAP EcoRI site was near the end of the injected fragment, it appeared to be intact in the genomic DNA of this transgenic animal. The 1.2 kb band represents the 3′-most region of the t-PA gene, which must have ligated head-to-tail to the 5′ end of the WAP gene, leaving the t-PA gene bounded on its 3′ end by an EcoRI site. Interestingly, the weak 2.3 kb band indicates that some of the copies of the fragments formed concatomers in a head-to-head configuration. KpnI digestion (lanes e–g) produced a single band of 4.9 kb, as expected. It is impossible to determine from this Southern blot whether all copies of the concatomer integrated at a single or at multiple sites.

Expression of biologically active t-PA in milk. Mice #wt1–26 and wt1–25 were mated to wild type males and had no apparent difficulty in conception or maintenance of pregnancy. Several days after parturition, milk was obtained from the females and was assayed for t-PA activity. Since wt1–11 was a male, it was necessary to obtain transgenic female progeny, mate them, and obtain milk from the second generation females after parturition. We have characterized expression from one progeny animal of this lineage, wt2–102. Since it was not known whether the mouse milk itself would interfere in the fibrin clot assay, we used standards consisting of recombinant t-PA added to milk from nontransgenic mice. As shown in Figure 2b, dilution of standards in milk did not affect the

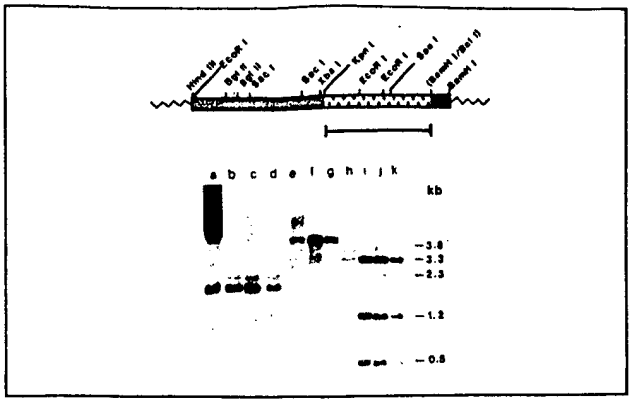

FIGURE 1 Generation of transgenic animals. Top portion: Restriction map of WAP-t-PA; Bottom portion: Southern blot of DNA from tails. Lanes a and h show 500 pg of WAP-tPA DNA digested with Sac I or EcoRI, respectively. Lanes b, c, and d contain 5 μg of DNA from mouse wt1–26, wt1–25 and wt1–7, respectively, digested with Sac I. Lanes e–g are from mouse tails of wt1–26, wt1–25 and wt1–7, respectively, cut with Kpn I, and lanes i–k are these DNAs cut with Eco RI. Lanes a–d were run on a separate gel than the rest of the lanes. Negative control DNAs did not show any hybridization to this probe under these conditions (not shown).

apparent concentration (in comparison to standards diluted in PBS), nor was there background clearing in the negative control sample wells. In this figure it can be seen that milk from wt1–26 cleared the fibrin clot to a significant extent. By comparison with lysis catalyzed by known amounts of added t-PA, the concentration was calculated to be about 200 ng/ml. In parallel assays, milk obtained from wt1–25 and wt2–102 was shown to contain 200 ng/ml and 400 ng/ml of t-PA (data not shown). When plates were incubated longer than 24 hours, minor clearing was seen in control wells containing milk from untransfected mice, but this was always significantly less than clearing seen from milk of any of the transgenic lineages. The origin of the residual fibrinolytic activity in nontransgenic mouse milk is not known. However, the presence of low levels of plasminogen activator (PA) in the lactating mammary gland of rodents[13] raises the possibility that some fibrinolytic protein is present naturally in milk.

Milk from mice wt1–26, wt1–25 and 2–102 was assayed by ELISA using an anti-human t-PA polyclonal antibody (Fig. 3). A standard curve was generated by addition of known amounts of human t-PA to mouse milk. Identical curves were generated by dilution of t-PA in cow milk and aqueous buffer (not shown). The inset of Figure 3 shows results of an assay of serial dilutions of milk from wt1–26 confirming that about 300 ng/ml of t-PA was present in this sample. Milk from wild type mice showed no signal in the ELISA. Milk from wt1–25 and wt2–102 contained t-PA at concentrations of 114 and 460 ng/ml, respectively (data not shown). The measurements by fibrin clot lysis and ELISA were not sufficiently accurate to determine precisely the specific activity of the t-PA produced in milk. Further studies of the purified protein (now in progress) will establish whether the specific activity of the protein is identical to that produced by melanoma cells and by recombinant DNA methods. Pilot studies indicate that t-PA remains stable and bioactive in whole milk for at least 48 hours at 37°C, and can be stored at −80°C (data not shown).

Since WAP RNA constitutes as much as 15% of the poly A (+) mRNA in the lactating mammary gland, it is probable that the level of t-PA in the milk of these mice is far below the level of endogenous whey acid protein. This

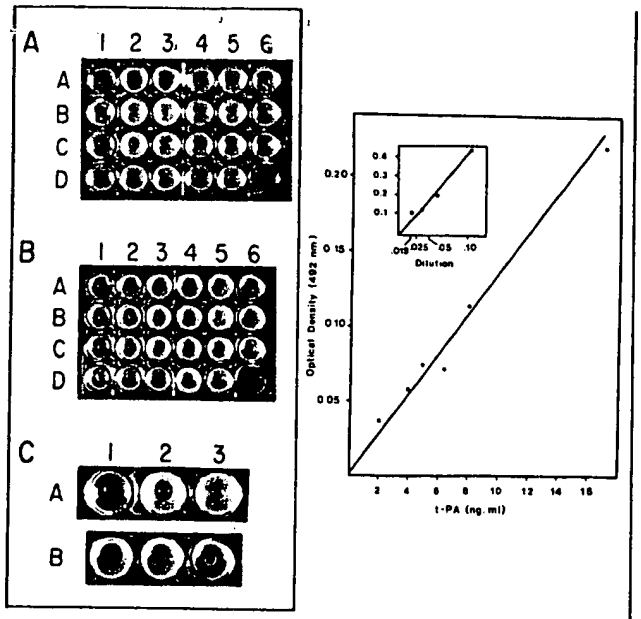

FIGURE 2 Clot lysis bioassay of secreted t-PA. (A) Transient expression of WAP t-PA in MCF7 cells. Two transfections were done, #1 (two repetitions, a and b) and #2 (3 repetitions, a, b, and c). In row A, columns 1 through 6 are recombinant DNA t-PA standards diluted in culture medium at concentrations of 20, 10, 5, 2.5, 1.25, and 0 ng/ml. Row B contains samples from transfection #1. In columns 1, 2, and 3 are three dilutions of sample from one transfection: 1×, .5×, and .25×, respectively. In columns 4, 5, and 6 or row B are similar dilutions from the repeat transfection. Row C, columns 1–3, row C, columns 4–6, and row D, columns 1–3 contain similar dilutions from the three repetitions of transfection #2. (B) Milk from transgenic mouse #wt1–26. Following identification of mouse #wt1–26 as a positive transgenic, the mouse was mated to a wild type male. Seventeen days after the first litter was born, milk was removed from the lactating female following stimulation with oxytocin. Milk was diluted in PBS by 50% and stored frozen. Milk was diluted further in PBS as indicated below just prior to assay and added to the wells of a fibrin clot lysis plate. The positive controls were generated by addition of recombinant t-PA to media composed of either 10% negative mouse milk (row A), 10% negative cow milk (row B), or PBS (row C). Concentrations of t-PA in the milk dilution curves, from columns 1 through 5 are: 40, 20, 10, 5, and 0 ng/ml. The concentrations in the PBS dilution curve, from column 1 through 6 are: 40, 20, 10, 5, 2.5, and 0 ng/ml. In row A, column 6 is the milk from mouse #wt1–26 at a final concentration of 10% and in row B, column 6 is the milk at a concentration of 5%. This photograph was taken after approximately 8 hours of assay incubation time. The negative mouse milk used for these controls was pooled from outbred CD-1 mice in different stages of lactation. In other negative controls (not shown) milk was used from inbred females of the same strain used for microinjection and was obtained at the same stage of lactation as the positive sample. The specificity of recombinant t-PA secreted into mouse milk was shown to be plasminogen in other experiments (not shown) in which plasminogen was omitted from the agarose matrix in similar fibrin clot lysis assays. (C) Enlargement of key data of sections A and B. Row A shows an enlargement of the data of section A (above), row C, columns 1–3; Row B shows one of the data of section B (above), row A, columns 4–6.

FIGURE 3 Quantitation by ELISA of recombinant t-PA secreted into milk of mouse wt1–26. The standard curve was performed in negative mouse milk diluted to a final concentration of 10% with PBS, to which was added t-PA supplied with the kit as indicated. The inset shows milk from mouse #wt1–26 in dilutions as indicated. The dilution of .1 refers to a final concentration of 10% milk. In each dilution of transgenic milk, samples were supplemented with negative mouse milk in order to keep the final concentration at 10%. All points of the control curve and the experimental (inset) curve have the background value (the value determined for negative mouse milk) subtracted.

could be due to many factors. Preliminary data indicate that variation in t-PA expression levels among transgenic mice containing WAP-tPA may be considerable, suggesting that the chromosomal integration site may play a key role in establishing levels of expression from this construction. In fact, one transgenic mouse (not shown) appeared to express virtually no t-PA in the milk. Thus, analysis of additional animals may identify those which produce more t-PA. In addition, intragenic and/or noncoding 5′ and 3′ sequences from the WAP gene, missing from the construction introduced into mice in these experiments, may play important roles in RNA stability. Considerable work remains to be done to configure the t-PA expression vector for maximal expression.

We demonstrate here that a foreign protein, human tissue plasminogen activator, can be secreted into the milk of transgenic mice under the control of a mammary-specific promoter. Thus, concerns that foreign proteins produced in the mammary gland might not be secreted, accurately processed, or be sufficiently stable in milk appear to be reduced by these results. The advantages of producing foreign proteins in this manner include the fact that milk is well characterized biochemically and that many of the genes encoding key milk proteins have been cloned. In addition, many milk-specific genes are expressed in the lactating mammary gland at high levels under hormonal control and in a tissue-specific manner. Thus, with expression cassettes similar to the one described here, it should be possible to target precisely foreign gene expression to the lactating mammary epithelium. Factor IX and t-PA have been produced in the blood of transgenic mice[14,15]; the ability to produce these proteins in milk would facilitate their collection. The ultimate goal of our experiments is to express foreign proteins in the milk of farm animals. Since production of transgenic farm animals has been achieved[16], this presents a reasonable possibility. Although many technical hurdles remain, the data presented here demonstrate that transgenic animals may become an attractive alternative for future production of genetically engineered biologically active proteins.

EXPERIMENTAL PROTOCOL

Construction of expression vector. A Hind-III site was added at the 5′ end of the 2.6 kb WAP promoter sequence (▭ -Ref. 5) by digestion at the single EcoRI site in the WAP promoter, filling in with Klenow and dNTPs, and ligation of Hind-3 linkers, all by standard protocols. The t-PA cDNA (▭)-SV40 termination/polyadenylation (■) cassette (see Ref. 11) was inserted into the polylinker region of the WAP promoter vector as a KpnI-Bam HI fragment.

Generation and analysis of transgenic animals. To purify the eukaryotic sequences for microinjection, WAP-tPA was digested with Hind-3 and BamHI, the fragments separated by gel electrophoresis, and the 4.9 kb fragment purified by binding to glass filter fiber papers[17]. After elution and concentration by ethanol precipitation, the DNA was suspended for microinjection in 10mM Tris, .05 mM EDTA, pH 7.5 at a concentration of .5 ng/microliter. The regulatory/coding restriction fragment from pWAP-tPA was microinjected into one cell fertilized embryos as described previously[18]. At four weeks of age, tail sections were taken from mice born from these injections, digested with proteinase K, phenol-chloroform extracted, then digested with various restriction enzymes. DNA was electrophoresed on a Tris-borate gel, blotted to nitrocellulose, and hybridized with a probe consisting of the entire coding region of t-PA cDNA (see bold line under the restriction map of Fig. 1). Labeling was done by extension of random hexamers to a specific activity of 1×10^9 cpm/μg.

Calcium phosphate transformation. MCF7 cells were plated in 100 mm dishes at densities of 5×10^6 or more per dish at least one day prior to transfection. Transfections were performed as described previously[19] and transient supernatants collected 48 hours after transfection were assayed for t-PA.

Fibrin clot lysis assay. The fibrin clot assay measures the ability of t-PA to digest fibrinogen matrices which are laid down

in a background of agarose, thrombin and plasminogen within the wells of a plate [20]. A small hole is bored through the agarose mixture upon hardening and 25 microliters of the samples are loaded into each of the holes. As t-PA diffuses into the agarose, clearing of the fibrinogen is evident visually and the amount of clearing is directly proportional to the amount of active t-PA. These assays are extremely sensitive and reproducible.

ELISA assay. Assays were performed with the IMUBIND ELISA kit produced by American Diagnostica Inc. The assay is a double antibody sandwich in which the primary antibody is a goat antiserum raised against t-PA from human uterus and the second antibody is a peroxidase conjugated anti-t-PA IgG. The standard curves were performed in negative mouse milk diluted to a final concentration of 10% with PBS, to which was added t-PA supplied with the kit.

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Phylogeny of Tamm-Horsfall Protein

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Uromodulin

Abstract
The Tamm-Horsfall protein (THP, uromodulin) is urine's most abundant protein. The phylogenetic distribution of the gene for THP was examined in all the major vertebrate classes. Genomic DNA was obtaind from each vertebrate class and hybridized with a rat THP cDNA probe in a DNA hybridization assay. In separate experiments, a polymerase chain reaction assay was used to amplify segments of the THP gene using primers from a consensus sequence for THP among multiple mammalian species. Both methods revealed that the THP gene was present in all the vertebrate classes. These data demonstrate the evolutionary conservation of the THP gene among vertebrates and suggest a role for this protein that is common to the kidney of all vertebrates.

Introduction

The Tamm-Horsfall protein (THP, uromodulin) is the most abundant protein in urine [1–3]. It is synthesized in renal tubular cells of the thick ascending limb of the loop of Henle [4–6]. It has been postulated to be important in the tubular transport of ions and in the pathogenesis of acute renal failure and renal stones [2]. The cDNA sequences for human [7, 8], bovine [9], rat [9, 10] and mouse [11] THP have been described.

Phylogenic distribution of THP has not been studied extensively. Two studies that have examined this issue, using immunohistology with polyvalent antiserum, have reported conflicting results. In the present study we investigated the phylogeny of THP by examining the presence of the THP gene in the major classes of vertebrates by DNA hybridization assay and by a polymerase chain reaction (PCR) assay.

Materials and Methods

Preparation of Genomic DNA

Liver tissue was obtaind from: zebra fish, *Danios sp.*; tree frog, *Hyla sp.*; turtle, *Chrysemys sp.*; white leghorn chicken, *Gallus sp.*; American opossum, *Didelhis sp.*, and white rat, *Rattus sp.* Tissue was frozen rapidly in liquid nitrogen and then crushed using a mortar and pestle in liquid nitrogen into a powder. For DNA extraction 1–5 g of powdered tissue was placed in 5 ml of extraction buffer (10 m*M* Tris-HCl, pH 8.0, 0.1 *M* EDTA, 0.5% SDS) containing 100 µl of a 10-mg/ml proteinase K for each gram of tissue and incubated with shaking at 56 °C for 96 h. DNA was precipitated from the supernatant by adding 13.8% vol of 5 *M* NaCl and 2 vol of 100% cold ethanol at −20 °C. DNA was removed by spooling on a glass rod, washed in 70% ethanol and dissolved in Tris-EDTA (10:1) buffer. DNA was deproteinized by repeated phenol/chloroform/isoamyl alcohol (25:24:1) extraction followed by ethanol precipitation until the OD_{260}/OD_{280} ratio was above 1.8. Any residual RNA was removed by incubating with 0.1% SDS and 1 µg/ml RNase at 37 °C for 60 min, followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Final DNA quantity was determined spectrophotometrically at 260 nm.

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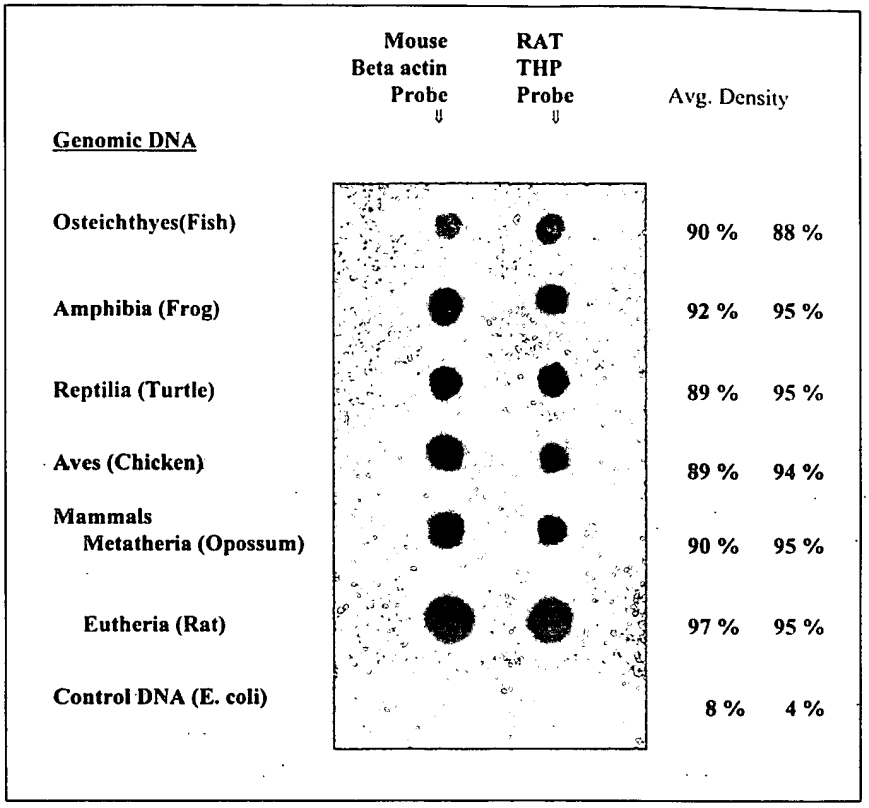

Fig. 1. Blot of genomic DNA from all vertebrate classes probed with rat Tamm-Horsfall protein (THP) cDNA and with mouse β-actin cDNA. 10 μg genomic DNA from each species was loaded in individual wells. DNA from all vertebrate classes was positive for THP gene; control DNA from *Escherichia coli* was negative.

DNA Hybridization Assay

DNA hybridization was performed on a nylon hybridization membrane (Gene Screen Plus, DuPont NEN Products, Boston, Mass.) using a 96-well manifold (BioRad Laboratories, Hercules, Calif.). The hybridization membrane was rinsed in a 0.4 *M* Tris HCl (pH 7.5) solution for 5 min and placed in the manifold. Samples of 20 μg genomic DNA from each species were denatured in a 100-μl solution of 0.025 *N* NaOH and 0.5 *M* NaCl for 10 min at room temperature, diluted to a total volume of 400 μl in 0.1 × SSC with 0.125 *N* NaOH and loaded on the membrane in duplicate wells of the manifold (10 μg DNA in 200 μl/well). The membrane was neutralized in 0.5 *N* NaCl with 0.5 *M* Tris-HCl (pH 7.5) for 10 min and the DNA fixed to the membrane by baking at 60°C for 1 h in a vacuum oven.

Membranes were removed from the manifold and prehybridized in an aqueous hybridization buffer containing 1% dextran sulfate, 1% SDS, and 5.8% NaCl for 2 h at 55°C. The membranes were cut in the center with each half representing one of the two sets of wells. One half of the membrane was hybridized with a 2.2-kb rat THP cDNA probe and the other half was hybridized with a 1.5-kb mouse β-actin cDNA probe. The probe solution was prepared containing 25 ng THP or β-actin DNA, radioactive α^{32}P-dCTP label, and salmon sperm DNA at a final volume of 100 μl. The probes had a specific activity ranging from 2–6 million cpm/ng and the hybridization was done overnight at 55°C. Membranes were washed once in 100 ml 2 × SSC, 1% SDS at room temperature, twice in 500 ml 2 × SSC, 1% SDS at 55°C for 30 min, and finally in 1 liter 2 × SSC, 1% SDS at 55°C for 2 h. After the final wash, the damp membrane was wrapped in plastic wrap, exposed to Kodak X-OMAT AR imaging film at room temperature for 48 h and developed.

Polymerase Chain Reaction Assay

Primers. The cDNA sequences of human, bovine, rat and mouse THP were compared. The THP gene has 10 exons of which exon 2 is the largest. Primer sets 1 and 2 were constructed by determining areas of greater than 95% homology in the second exon among these species. Primer set 1 amplifies a 248-bp fragment between position 449 and 697 in the mouse THP cDNA sequence and primer set 2 amplifies a 179-bp fragment between positions 518 and 697 in the mouse THP cDNA sequence. For controls, β-actin primers were obtained from Stratagene (San Diego, Calif.; Catalog No. 302010) that amplify a 661-bp fragment. One microgram DNA from each species was amplified by PCR using recombinant Taq polymerase (AmpliTaq, Perkin Elmer, Foster City, Calif.) at 2 m*M* $MgCl_2$. Thermal cycling conditions were: denaturation at 95°C for 120 s, anneal-extend period of 35 cycles, 60 s each at 60°C for β-actin and at 56°C for THP, and a final extension at 72°C for 7 min. The total volume was 100 μl for each reaction. Five-microliter aliquots of each of the two reactions (THP and β-actin) for each species were combined and run on a 1.4% agarose gel, stained with ethidium bromide and photographed under ultraviolet light.

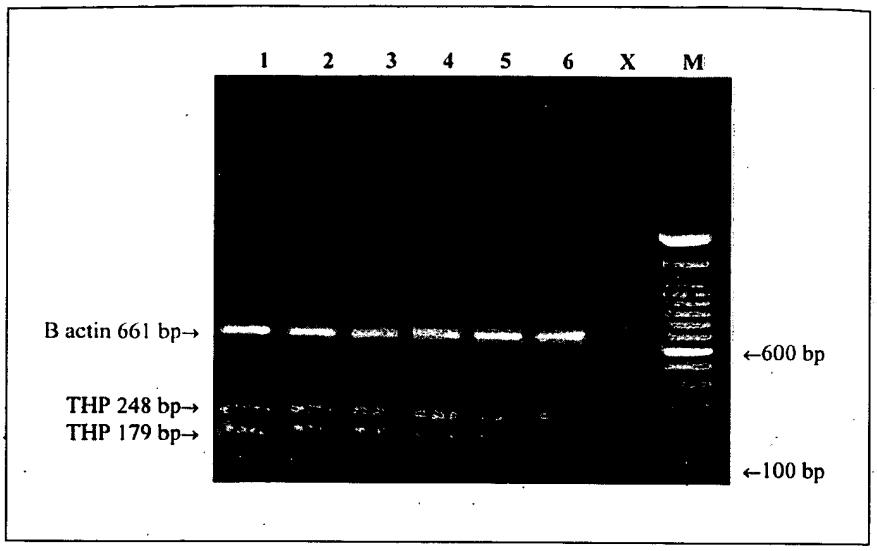

Fig. 2. Presence of Tamm-Horsfall protein (THP) and β-actin gene in various vertebrate classes by polymerase chain reaction (PCR). The products of three separate PCR reactions, two using THP-specific and one using β-actin-specific primers were combined for each species. Hence, the single β-actin amplification product (661-bp size) and two THP amplification products (248- and 179-bp sizes) for each species are shown in the same lane. Lane 1 = Osteichthyes (zebra fish); lane 2 = Amphibia (frog); lane 3 = Reptilia (turtle); lane 4 = Aves (chicken); lane 5 = mammalian subclass Metatheria (opossum); lane 6 = mammalian subclass Eutheria (rat); lane X = control lane with no template DNA; lane M = marker DNA.

Results and Discussion

The DNA hybridization (fig. 1) showed the THP gene to be found in the following classes: Osteichthyes (zebra fish), Amphibia (frog), Reptilia (turtle), Aves (chicken), and Mammalia subclasses, Metatheria (opossum) and Eutheria (rat). The mouse β-actin gene, used as a control, was also demonstrated in each species studied. DNA from *Escherichia coli* was used as a negative control and showed only a background reaction for both THP and β-actin.

PCR assay of genomic DNA (fig. 2) using two sets of primers also showed the THP gene to be found in the following classes: Osteichthyes (zebra fish), Amphibia (frog), Reptilia (turtle), Aves (chicken), and Mammalia subclasses, Metatheria (opossum) and Eutheria (rat).

These data show that the gene for THP is evolutionarily conserved and is present in each of the vertebrate classes. The phylogeny of THP has previously been examined in two studies, both using polyvalent antiserum to detect the presence of THP in assorted tissues. Wallace and Nairn [12] found THP present only in the kidneys of placental mammals but not in marsupial or monotreme mammals or in other vertebrate classes including fishes, birds, amphibians and reptiles. Howie et al. [13, 14] found THP in the kidneys of all mammals, in the kidney and skin of the vertebrate class Amphibia, and in the skin and gills of the vertebrate classes Chondricthyes and Osteichthyes. The differences between these studies are presumably due to differences in specificity and cross-reactivity of the polyvalent sera used and the sensitivity of the histological techniques. Our data extend these previous data by demonstrating, for the first time, the universal presence of the THP gene in all vertebrate classes. These findings suggest a function for THP that is common to the physiology of all vertebrate kidneys.

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Cloning and sequencing of the CRABP-I locus from chicken and pufferfish: analysis of the promoter regions in transgenic mice

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Retinoic acid (RA), a derivative of vitamin A, is an important molecule for development and homeostasis of vertebrate organisms. The intracellular retinoic acid binding protein CRABP-I has a high affinity for RA, and is thought to be involved in the mechanism of RA signalling. CRABP-I is well conserved in evolution and shows a specific expression pattern during development, but mice made deficient for the protein by gene targeting appear normal. However, the high degree of homology with CRABP-I from other species indicates that the protein has been subject to strong selective conservation, indicative of an important biological function. In this paper we have compared the conservation in the expression pattern of the mouse, chicken and pufferfish CRABP-I genes to substantiate this argument further. First we cloned and sequenced genes and promoter regions of the CRABP-I genes from chicken and the Japanese pufferfish, *Fugu rubripes*. Sequence comparison with the mouse gene did not show any large blocks of homology in the promoter regions. Nevertheless, the promoter of the chicken gene directed expression to a subset of the tissues that show expression with the promoter from the mouse gene. The pattern observed with the pufferfish promoter is even more restricted, essentially to rhombomere 4 only, indicating that this region may be functionally the most important for CRABP-I expression in the developing embryo.

Keywords: CRABP-I; chicken; pufferfish; promoter region; transgenic mice.

Introduction

Retinoic acid (RA) exerts a wide variety of effects on vertebrate development, cellular differentiation and homeostasis. Both excess and deficiency of RA during embryonic development result in a spectrum of congenital malformations (Wilson *et al.*, 1953; Kochhar, 1967; Lammer *et al.*, 1985). The effects of RA are mediated at the molecular level via two classes of proteins, the retinoic acid receptor family and the retinoic acid binding proteins. The retinoic acid receptors are nuclear ligand-inducible transcriptional regulators belonging to the nuclear hormone receptor superfamily (Leid *et al.*, 1992; Mangelsdorf *et al.*, 1995; Chambon, 1996). The cellular retinoic acid binding proteins (CRABP-I, CRABP-II) are small binding proteins with high affinity for all-trans RA. In the adult organism CRABP-I is widely expressed, whereas CRABP-II expression is restricted to the skin. Both proteins show spatio-temporally specific expression patterns during embryonic development (Dolle *et al.*, 1990; Ruberte *et al.*, 1991; Maden *et al.*, 1992; Ruberte *et al.*, 1992; Lyn and Giguere, 1994). Expression of CRABP-I is found in those tissues that are most sensitive to excess of RA, notably the developing central nervous system, the neural crest, limb bud mesenchyme and mesenchyme in the frontonasal mass and branchial arches (Vaessen *et al.*, 1990). Although the protein is mainly localised in the cytoplasm, recent reports have shown that in certain cell types CRABP-I can be found in the nucleus, suggesting a possible role in nuclear import of RA (Gustafson *et al.*, 1996). A number of other roles have been suggested: it could act as a cytoplasmic buffer, protecting sensitive cells against excess of RA, it might be involved in the metabolism of RA to polar metabolites, or it could, under conditions of vitamin A shortage, function to sequester RA in the cells that are most dependent on it (Donovan *et*

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al., 1995; Napoli, 1996). However none of these roles have been satisfactorally proven. Moreover, mice deficient in CRABP-I or both CRABP-I and II are essentially normal, showing that CRABP-I is dispensible for normal development, at least under laboratory conditions (de Bruijn *et al.*, 1994; Gorry *et al.*, 1994; Lampron *et al.*, 1995). In sharp contrast to this stands the fact that CRABP-I is highly conserved in evolution, which would indicate that it has functional importance for the organism. Bovine and murine CRABP-I are completely homologous at the amino acid level, and differ in only one out of 136 amino acids from human CRABP-I (Sundelin *et al.*, 1985; Vaessen *et al.*, 1989; Astrom *et al.*, 1991). From a partial chicken cDNA it can be seen that chicken and mouse CRABP also share a high degree of homology at the amino acid level (Vaessen *et al.*, 1990). Thus it appears that, even though the protein is apparently dispensible for mice under laboratory conditions, even a small change in the sequence of the protein is evolutionary unfavourable. Here we further substantiate this point with the cloning and sequencing of the CRABP-I genomic loci from chicken and the Japanese pufferfish, *Fugu rubripes*. Sequence analysis of the promoter regions from both these species was carried out, with the aim of identifying conserved promoter/enhancer elements. We have recently shown that the expression of CRABP-I in the mouse embryo is regulated via distinct sets of enhancer elements (Kleinjan *et al.*, 1997). Expression in the neural subdomain of its expression pattern was shown to be regulated through elements located well within a 3 kb upstream fragment. The expression pattern of CRABP-I in the chicken embryo is similar to that in the mouse, suggesting that the same *cis*-acting transcription elements may be involved in the regulation of expression. Although no information is available on the expression of CRABP-I in the pufferfish, its small genome size makes it a useful model species to look for conserved regulatory elements (Brenner *et al.*, 1993; Aparicio *et al.*, 1995). Here we report the cloning and sequencing of over 3 kb of upstream promoter sequences from both of these species. These sequences were tested for transcriptional regulatory activity in transgenic mice, where they are shown to drive specific patterns of expression of a *lac*Z reporter gene in the hindbrain region. The sites of *lac*Z expression form a subdomain of the murine CRABP-I expression pattern, with the extent of the subdomain decreasing with increasing evolutionary distance between the species.

Materials and methods

Cloning, mapping and sequencing of CRABP-I from Fugu rubripes

The clones A176 and K1328 were isolated from a gridded ICRF *F. rubripes* genomic cosmid library, based on the cosmid vector Lawrist4 (C. Burgtorf and H. Lehrach, Berlin). The library was screened using a ^{32}P-labelled probe generated from the 170 *Taq*I fragment from mouse CRABP-I (Vaessen *et al.*, 1990), in a hybridization mixture containing dextran sulphate (Sambrook *et al.*, 1989). Duplicate nylon filters were hybridized at 56 °C for 16 h, washed twice for 20 min in 3 × SSC/0.1% SDS, and once in 1 × SSC/0.1% SDS (1 × SSC = 150 mM NaCl/15 mM sodium citrate, pH 7) at 56 °C, and autoradiographed. Positive clones were picked and grown for DNA isolation. The cosmid clones A176 and K1328 were used to map the *Fugu* CRABP-I locus. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridisation were performed as described (Sambrook *et al.*, 1989). Various fragments were subcloned into pBluescriptII for sequencing. The genomic sequences of the upstream region and surrounding the exons have been submitted to the EMBL database under accession numbers Y12240, Y12241 and Y12242.

Cloning, mapping and sequencing of CRABP-I from chicken, Gallus gallus

The phage clones ChCRABP 8 and ChCRABP 17 were isolated from an adult Leghorn Chicken liver EMBL-3 SP6/T7 genomic library (Clontech) using a 310 bp chicken CRABP-I partial cDNA clone as probe (Vaessen *et al.*, 1990). Hybridization of the library was performed as described for the *Fugu* library, except that in this screening the filters were hybridized and washed at 65 °C. Positive plaques were rescreened and positive clones were used to isolate DNA. Mapping and sequencing of the chicken CRABP-I locus was done as described for *Fugu* CRABP-I. Genomic sequences of the upstream region, and surrounding the three sequenced exons have been submitted to the EMBL database under accession numbers Y12243 and Y12244.

Production and analysis of transgenic embryos

The microinjection constructs containing the mouse, chicken and pufferfish promoter regions were made as follows: A 5500 bp genomic *Eco*RI fragment containing exons I and II of the murine CRABP-I gene was subcloned and in the resulting plasmid the C residue at position +4 of the coding sequence was changed to a G by site-directed mutagenesis, thus creating an *Nco*I site at the translational start site of the gene. A 30 bp sequence coding for a 10 amino acid peptide tag derived from the human c-*myc* proto-oncogene (Evan *et al.*, 1985) was cloned into this site, to create pDJTag. pECTag was constructed by linking the cDNA sequences for exons II, III and IV in frame to exon II of pDJTag. The mouse promoter/*lac*Z reporter construct was created by inserting an *Eco*RI/*Xho*I upstream fragment from the murine CRABP-I gene into the vector p610Za (Kothary *et al.*, 1989), resulting in EXp610Za. The vector p610Za

contains a *lac*Z gene driven by a mouse heat-shock promoter (hsp68). It has been demonstrated that this hsp68-*lac*Z construct does not give any constitutive expression in transgenic mouse embryos, making it a useful vector for testing for the presence of regulatory elements in heterologous sequences (Kothary *et al.*, 1989). The microinjection fragment BXhspZ was isolated by *Bgl*II/Asp718 digestion of EXp601Za.

The chicken promoter/reporter construct Ch1 was created by subcloning a 6 kb *Eco*RI fragment containing exons I and II into pBluescriptII KS. From this plasmid, a 3.6 kb *Not*I fragment containing the chicken upstream and minimal promoter region, exon I, intron I and part of exon II was excised and ligated into the IRES-LacZ vector βGeo (Mountford *et al.*, 1994). The *Fugu* promoter reporter/construct Fu1 was made by subcloning of a 4.2 kb SpeI fragment, containing the *Fugu* promoter region as well as exon I and part of intron I, from cosmid A176 into pBluescriptII KS. From the resulting construct, a 3.8 kb *Xho*I fragment was subcloned into the *Sal*I digested p610Za vector.

Microinjection of mouse oocytes was performed according to standard procedures (Hogan *et al.*, 1994). Primary embryos were isolated from pregnant females at day 10.5 or 11.5 p.c.. For *lac*Z staining the embryos were fixed in 1% formaldehyde, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM ethylene glycol-bis(beta-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), 0.02% Nonidet P-40 (NP-40) in PBS (phosphate-buffered saline) for 60–90 min, washed with PBS containing 0.02% NP40, and stained for several hours or overnight at 37 °C in the dark in a solution containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ $3H_2O$, 2 mM $MgCl_2$, 0.01% sodiumdeoxycholate, 0.02% NP40 and 0.1% 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-gal).

Embryos transgenic for the ECTag construct, containing the Myc-tag as a reporter, were fixed for 1 h in 35% methanol, 35% acetone, 5% acetic acid. After dehydration in an ethanol/xylene series the embryos were embedded and sectioned. The sections were rehydrated in ethanol/xylene. Aspecific binding was blocked by pre-incubating the sections in PBS/Tween20 (0.05%) containing 2% NGS (normal goat serum). After three washes with PBS/Tween20 the sections were incubated overnight at 4 °C with a monoclonal antibody agains the Myc epitope in a 1:100 dilution in PBS containing 1% BSA and 0.05% Tween20. After washing the sections were incubated with an alkaline phosphatase conjugated goat anti-mouse antibody for several hours at room temperature. The sections were washed and exposed to NBT/BCIP as described elsewhere (Sambrook *et al.*, 1989). Finally, the sections were dehydrated and mounted in glycerol/PBS.

Results

Cloning and characterisation of pufferfish CRABP-I

Using a 170 bp *Taq*I fragment encoding part of the mouse CRABP-I cDNA as a probe we have screened a gridded *F. rubripes* genomic ICRF cosmid library (C. Burgtorf and H. Lehrach, Berlin) under reduced stringency conditions. This resulted in the isolation of a small number of positive clones. Restriction mapping, hybridization with other probes and sequencing of a small fragment revealed that the isolated clones could be categorised into two groups derived from two different loci. Partial sequencing and hybridization with additional mouse CRABP-I cDNA probes identified the clones A176 and K1328, representing one group, as *Fugu* CRABP-I. The other group of clones, possibly representing CRABP-II, was not further characterized. A restriction map of cosmid A176 is shown in Fig. 1A. As all other CRABPs, the gene contains four

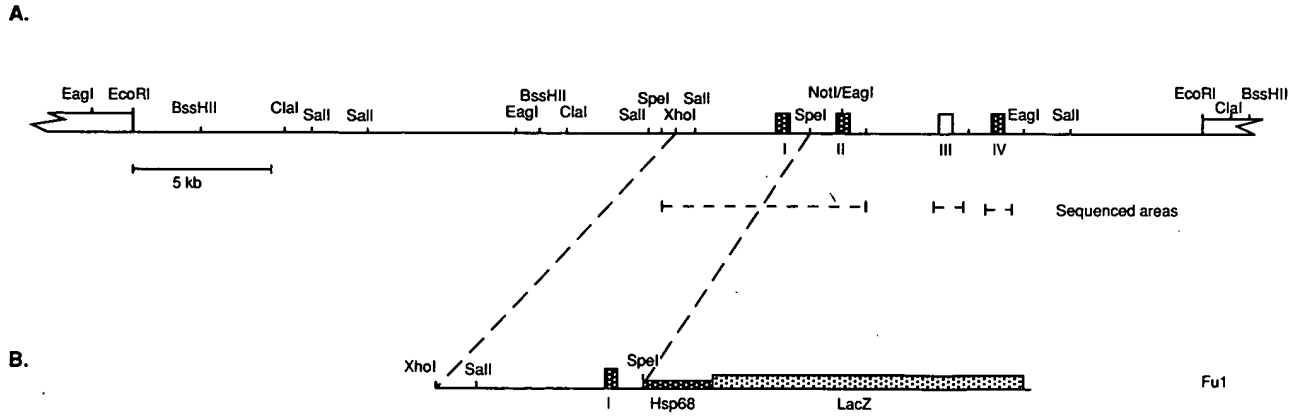

Fig. 1. (A) Map of cosmid A176, containing the CRABP-I locus from the Japanese puffer fish, *F. rubripes*. The 4 exons are indicated as filled boxes. The regions that have been sequenced are indicated by the broken line underneath the map. (B) Fu1 is the microinjection fragment that has been used to assess the puffer fish promoter region in transgenic mice. It contains 3 kb of upstream sequences, exon I and part of intron I driving a hsp68 promoter/*lac*Z reporter cassette.

exons. The overall size of the gene is approximately 9 kb, and therefore no smaller than the mouse gene. To determine the sequence of the gene and the promoter region, fragments spanning the regions indicated in Fig 1A were subcloned into BluescriptII vectors for sequence analysis. The sequences of the four exons, and deduced amino acid sequence are presented in Fig. 2. Comparing the coding sequences of the exons of the *Fugu* gene with those of the mouse revealed a 78% sequence homology, and indicated that the intron/exon borders have been conserved. All splice donor and acceptor sites comply with the consensus GT and AG rule (Breathnach and Chambon, 1981). At the amino acid level the deduced pufferfish CRABP-I protein is 86% homologous to mouse CRABP-I.

Cloning and characterisation of chicken CRABP-I

To clone the genomic chicken CRABP-I locus we used the 310 bp partial chicken CRABP-I cDNA clone ChCRABP C4.5 (Vaessen *et al.*, 1990) as a probe to screen an EMBL3 SP6/T7 Chicken genomic phage library (Clontech). We isolated a number of clones, none of which

```
   1  actagtgtga gtcaaaaatc agaacgaatg ttggtgttaa aatgttattc
  51  caatacaagt aaattcataa aatcccagcc ttgaatattg gtccacgctt
 101  acctggtcca gcatccatgg acttaatctg ttttccgagc tcgagatccc
 151  acagacggat gtgtgcatcc agggagctgg aggtcagcga tggctccatt
 201  gtgactgatg tcactgacac tactctcagc tggtgtctca gagtcatgnn
 251  ctgcaggcgc gatgtggggg aaagataccc gacgttcagg gcaaattaga
 301  tcaacattca ggcaaaataa actgctgtag aatatcaaat tctactctta
 351  gtggtgatat taggtaagaa tctggacagt tttggataac tggtgccttt
 401  cacgataaat gactgattat caacagctca cagttactgg ttgtcattac
 451  gacaaagaga tacggtttct tttgaaacaa tgtttactgt ttacattgtt
 501  attggtattt tcagaaaaac caaagccact caccacattt ccagactttc
 551  acatgtcatc taatgaacct gtgatgatag tttctgtccc atctgcctca
 601  cttttacccc acgctgctgt ccagatagca tcatcgtggg ctaagaggga
 651  acacatgcac tcttcatccg tgttctctta atgttagcgc ttcaatacaa
 701  accatgcagt aaaaacaagc aggcctctat atggaaactg aaataaatgt
 751  ttccaaccta ccgtgttctt gcttgaaaag aatgcagtag ctgaaataaa
 801  agaaacagta acagtaaata ttttacccat aaaataggag gacgctcaaa
 851  atgaaagtga agttggttga acttgttgct tacttgagtg ctcatttttg
 901  gttctgagag tcacattcag tcagacagct aacctacaca ttaagcgttt
 951  tacattctgc accagctcct tgcaaatgta ttcatgttaa caacatgtta
1001  acacagtagg aatgggataa cattttgag tttaaccaac accatttag
1051  gtttaaccgg tattaatgtg aggagaattt ccacatgagt aatagcattt
1101  tagagatttc tagacattta tgaccagaac aaactgccag ttttttaatc
1151  tctttgctgg ttgaagtacc attgtgaaat ataaacttta gcaggagcta
1201  aagtcgactg acagcttagc gctacattga tcacagacaa tgaaacccag
1251  tcagcagatc tagattatcg cctcgtttaa gaagcgaaca atgctgtatg
1301  gtgacgattg tatcatttaa atatatccaa taaatataaa tattgaccca
1351  taccttgaat tcagctagta tggggtttct tcgattaaga gttcccgtgt
1401  attcataagg gagaagccat aacacgacga aacttcggtt tgttcttctt
1451  cgtttctata ccagttaact aataacgcac agagctctac tgccaccagt
1501  ggacaaaaag cgtttgcttc attaggaaaa ttggtaattg agttgtttcg
1551  ctattactgt attacaaaat tattcaatta catttttag gataatatta
1601  acaatattac acactaatca gtgatgattg ttcgtaatca ttctgtttac
1651  attattatgt atttgattat atagtcactg ttatgcagtc tgtcgcagat
1701  agagtgtatg tcaatctatt gcgcgtaatt tatcttacag tcgtgtgcag
1751  aaaaaatgta aatgtatctt tacagtctgt gagcaagtaa agggccgggg
1801  aaccacactg agggtgtggt ttcctgatca ggctgtgatg atgaactgca
1851  acggagggcc cacccaacct gaaaacgggt aaacaagcag acaacaacag
1901  gatcacacaa cgtgaggaat ccctaacaga cagctctgcg acttaagccc
1951  ggcttaaata aagtgtcttg ttaaataatg atcattttaa aaaaaaaatc
2001  cttttgcatg ataagttaag ctaaaaatct gttcttcaga gactgatgtt
2051  ggatatgagg atgcaaaaat ttgtaaaggc ttttaaactg ttatttctat
2101  ttcctagctg aaatcatctg gagatatttt cacaatccaa gtaaaaaatc
2151  cactaaaaag ttcagactca gtctaacttc gctggtgagc ttcagcacac
2201  acatctttca tctatttttg taatgctgca ttgtgacaat aaattttaat
2251  aggaggacaa ctttttttta aagttgtgtc tgcaaaaaga atacagttga
2301  tataatcttc cttaatcacc attaaattat atatgtacac tgcaaaaata
2351  tgtaaagtgt gaattacaaa tgtacattgt attctttagt aggactggca
2401  gaatgccaaa cattttgtat tttgcaaaaa taaattacct gccaattttt
2451  tttaatctgt ttttcttatc tgtttctata gaaatgacac aatattgatg
2501  acaatcaatg caaaacctac agttttgccc ccctgtactc atagggcagc
2551  atttttggga ggggtgggtg gccctcctcc cattgagacà gtagctataa
2601  agcgagactg ctggtgtact gagagcgctg tagaggtgag agcgagtgag
2651  gcagaaatat acatttacag gacacccaga aacacacact tgcattttaa
2701  gagtgtgatt ccagagcgga ggacaggagc acagggaagc aggaggagag
2751  gtgagaaaga gatcagagag agcacgaaga cggcagcaag cagtcacaac
2801  tacgcgtgtg gtgagacacg agcgtccttc aagcgacaga gctcctcgcg
2851  cgctcagacg ctctagacga gaacaactag ATGCCAAACT TTGCCGGCAC
                                     M  P  N  F  A  G  T
2901  CTGGAAGATG AAGAGCAGTG AGAATTTTGA TGAACTTCTC AAAGCCTTGG
        W  K  M  K  S  S  E  N  F  D  E  L  L  K  A  L
2951  gtaagccctc tctcttcctc ctcttgattt ctcacaagca aatcctgtgc

      ------------------- 1.7 kb ---- intron I -------------

   1  ttttgttatt gattgaggga agataactga ccgactgggt ctcctgcctc
  51  ctctgtgctg ctcagGGGTC AACACCATGC TGAGGAAGGT GGCCGTGGCG
                    G  V  N  T  M  L  R  K  V  A  V  A
 101  GCCGCCTCCA ACCCGCACGT GGAGATCCGT CAGGACGGGG AGAAGTTCTA
```

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        A  A  S  N  P  H  V  E  I  R  Q  D  G  E  K  F  Y
151  CATCAAAACC TCCACCACGG TGCGCACCAC CGAGATCAAC TTCCACATCG
       I  K  T  S  T  T  V  R  T  T  E  I  N  F  H  I
201  GGGAAGAGTT TGACGAGGAG ACGGTGGACG GGAGGAAGTG TAAGgtaacg
     G  E  E  F  D  E  E  T  V  D  G  R  K  C  K
251  aatcttctcg agcccgatgg aggcaaaact gacaggttgg ggattcttta

     ------------------- 3.2 kb ---- intron II -----------

  1  gatcacattt gctctgtttg ttctgcccac agAGTCTCCC CACCTGGGAG
                                                S  L  P  T  W  E
 51  TCGGAGAACA AGATCCGGTG CAAGCAAACC CTGGTGGAAG GTGACGGCCC
       S  E  N  K  I  R  C  K  Q  T  L  V  E  G  D  G  P
101  CAAAACCTTC TGGACCCGGG AGCTGAACGG GGACGAGCTC ACACTGgtga
       K  T  F  W  T  R  E  L  N  G  D  E  L  T  L
151  gctctaaaaa cctgtgacga ctgctctaga gtccagtcac tgggagtgtt

     ------------------- 2.0 kb ---- intron III ----------

  1  cttccctctg ttcttccagG TTTTCGGGGC AGACGATGTC GTCTGCACAC
                           V  F  G  A  D  D  V  V  C  T
 51  GAATTTACGT CCGTGAATGA agctctgtgg cctccttcag acaaccgggt
     R  I  Y  V  R  E  *
101  agaagcccac ctgaccccac cgcgttcaaa cccatcgaca ttctgtctcg
151  ctaacgtgct ggcgaatatg ccaagtgttc cgtgtgggtc tgcttagtgt
201  tcgcgtgcag tagaaatgtc tcagtacttc ttaggtgcag gactgacggt
251  cctggaacca gctgtttatg taggttagtt ccagacgtac tcattagcta
301  ttcatgtcca gtgacgcgaa ataaaaggag aggagcaaca gtgtctcggc
351  ttctttattc taaaataaac tgatgttaaa ggcaaagaaa ttgaccctac
401  tcacctgtcc tgatgtgacc tcagcagatg cctggtctat gagaggtttc
451  ataccgctgg gtctgaatgt gttccctaac acaccttcag tcaggagctg
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Fig. 2. Genomic sequences from the CRABP-I locus from puffer fish, *F. rubripes*. The coding sequences are written in bold face, and the deduced amino acid sequence is indicated underneath in one letter code. The introns have not been fully sequenced and are indicated by a broken line. Two putative polyadenylation signals are underlined.

contained the whole gene. Restriction mapping and hybridization with probes specific for mouse exons I, III and IV showed that phage clone 8 contains the 5′ end of the chicken CRABP-I locus, and phage clone 17 contains the 3′ end (Fig. 3B). A restriction map of the chicken CRABP-I locus can be drawn from the combination of the two phages as shown in Fig. 3A. Fragments spanning the regions indicated in Fig. 3B were subcloned into BluescriptII. We have determined the sequence of the first three exons and 3 kb of the promoter region. The sequence of these exons, and their deduced amino acid sequence are presented in Fig. 4. The fourth exon has been located on an 8 kb *Hin*dIII fragment, but attempts to sequence it using a mouse exon IV primer were unsuccesful. The sequence of exons I, II and part of III is identical to the partial cDNA sequence found by Vaessen, and differs in two residues from the published partial cDNA isolated by Maden, both of which are located in the PCR primers used to isolate their clone (Vaessen *et al.*, 1990; Maden *et al.*, 1992). Comparing the sequence of the first three exons of chicken CRABP-I with that of the mouse reveals an 86% sequence homology, and indicates that the intron/exon borders have been conserved. At the amino acid level the deduced chicken CRABP-I protein is 94% homologous to mouse CRABP-I. The homology between chicken and *Fugu* CRABP-I is 85% at the nucleotide level and 85% at the protein level.

Analysis of the promoter region of chicken CRABP-I

In addition to the first three exons of the gene we have also sequenced approximately 3.2 kb of promoter sequences of chicken CRABP-I, with the aim of identifying conserved *cis*-acting elements involved in the transcriptional regulation of the gene. Comparison with the known promoter sequences of the mouse gene did not reveal any homologous elements of significant size. Functional homology between the chicken and mouse promoter regions was assessed with a reporter construct containing chicken CRABP-I promoter sequences (Fig. 3C). The microinjection fragment derived from it, named Ch1, contains 3 kb of upstream sequences, exon I, intron I, and is linked in exon II to a *lac*Z reporter cassette containing an internal ribosomal entry site (IRES-*lac*Z cassette) (Mountford *et al.*, 1994). Three independent transgenic embryos were obtained with Ch1. All three embryos were

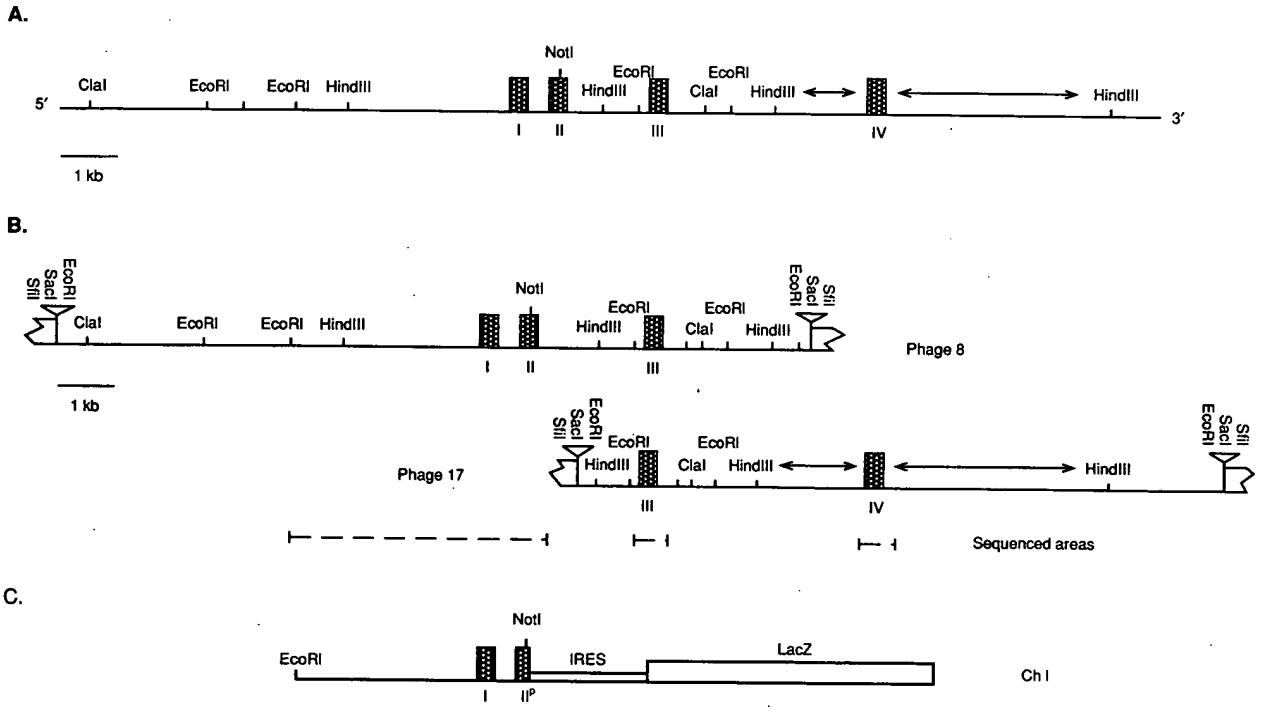

Fig. 3. (A) Genomic organisation of the chicken CRABP-I locus. (B) Phages 8 and 17 were obtained from screening a chicken genomic library. Phage 8 contains exons I, II and III, as well as 8 kb upstream sequences. Phage 17 contains exons III and IV of CRABP-I plus downstream sequences. The broken line underneath the map of the phages indicates the regions that have been sequenced, which include the first three exons of the gene as well as 3 kb upstream promoter sequences. The arrows around exon IV indicate that the exact position of exon IV has not been further determined. Not all *Eco*RI sites are indicated (C) Ch1 is the microinjection fragment that was used to assess the chicken CRABP-I promoter region in transgenic mice. It contains 3.6 kb of chicken CRABP-I sequences, including 3 kb of upstream sequences, the chicken minimal promoter, exon I, intron I and is linked in exon II to a *lac*Z reporter gene carrying an internal ribosomal entry site (IRES-*lac*Z reporter cassette).

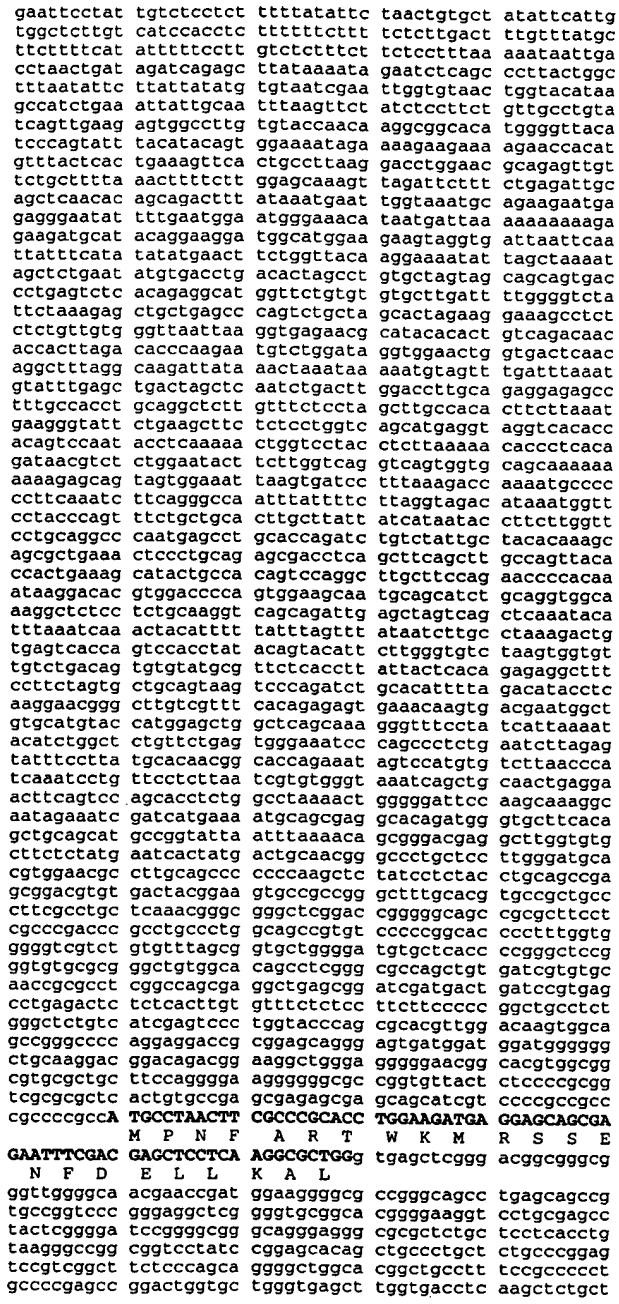

Fig. 4. Genomic sequences from the chicken CRABP-I locus. The sequences encoding the first three exons of the gene are written in bold face, and the deduced amino acid sequence is indicated underneath in one letter code.

consistent in showing specific *lac*Z staining in the developing hindbrain. *lac*Z expression in 10.5 day p.c. embryo was restricted to rhombomeres 2, 4, 5 and 6, and at lower levels in 7 and 8 (Fig. 5D) which is identical to a subdomain of the expression pattern of mouse CRABP-I in the hindbrain. In addition to the staining in the hindbrain some aspecific staining due to position effects was seen in two of the embryos (Fig. 5C). In one of the embryos a relatively weak *LacZ* expression, which is possibly due to the use of an internal ribosomal entry site-*lac*Z cassette, is largely overshadowed by strong ectopic expression. The presence of ectopic expression in these embryos shows however that the chicken minimal promoter is functional in transgenic mice.

When the similar 3 kb upstream promoter region from the mouse CRABP-I gene is used to drive expression of either a *lac*Z reporter gene, construct BXhspZ, or an epitope tagged version of CRABP, construct ECTag,

expression of the reporter gene is found not only in the hindbrain region, but also in the midbrain, in the cranial nerves and in the mantle layer of the neural tube (Fig. 5A). The pattern of expression reflects the neuronal subdomain of the endogenous murine CRABP-I expression pattern. The rhombomere specific pattern of CRABP-I expression in the hindbrain of the mouse is illustrated in a section through the head region of an embryo with construct ECTag, showing the expression of the transgene at low levels in rhombomere 2, at higher levels in rhombomeres 4, 5 and 6 and again at lower levels more caudally, in addition to expression in the midbrain and in the neural tube (Fig. 5B). Thus, the staining pattern shown by the chicken promoter transgene appears to form a subdomain of the expression pattern found with similar mouse promoter transgenes, BXhspZ and ECTag (see Materials and methods).

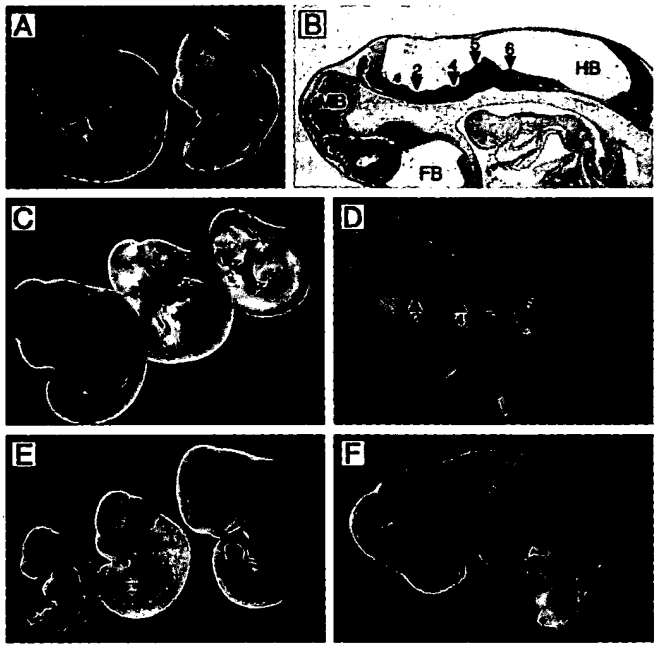

Fig. 5. Transgenic embryos obtained from oocyte injections with a *lac*Z reporter gene driven by upstream CRABP-I promoter regions from mouse (A, B), chicken (C, D) and puffer fish (E, F). The mouse upstream region drives expression of *lac*Z in the midbrain, hindbrain and neural tube. A section through the hindbrain region of a transgenic embryo, carrying an epitope tagged reporter gene (CRABP-Tag) driven by the mouse CRABP-I upstream region, ECTag, shows the specific expression in rhombomeres 2, 4, 5 and 6 of the hindbrain. (C) Transgenic mouse embryos with the chicken promoter region/*lac*Z construct Ch1, showing expression of *lac*Z in the hindbrain region. Expression in other sites is due to position effects and therefore non-consistent between the embryos. (D) A 9.5 day p.c. Ch1 embryo showing staining in rhombomeres 2, 4, 5 and 6. (E) Transgenic embryos obtained from injections with the puffer fish promoter region/*Lac*Z construct Fu1. Specific staining is found only in rhombomere 4. (F) In a 9.5 days p.c. embryos the rhombomere 4 staining is extended to the neural crest cells that migrate from this rhombomere to the second branchial arch. FB, forebrain; MB, midbrain; HB, hindbrain; OV, otic vesicle; numbered arrowheads indicate the positions of the rhombomeres.

Analysis of the promoter region of Fugu *CRABP-I*

As with the chicken gene we have also sequenced approximately 5 kb of promoter sequences of the *Fugu* CRABP-I gene, including 3 kb upstream and the complete first intron, to identify conserved regulatory elements. Again, we were unable to locate any homologous elements of significant size. Functional homology was assessed by a reporter construct containing *Fugu* upstream sequences (Fig. 1B). The microinjection fragment Fu1 contains 3 kb upstream sequences, as well as exon I and part of intron I, driving a Hsp68-*lac*Z expression cassette (Kothary *et al.*, 1989). Three independent transgenic embryos were obtained with Fu1. Interestingly, all three embryos showed specific *lac*Z staining in rhombomere 4 of the developing hindbrain (Fig. 5E). In the 9.5 day p.c. embryo the rhombomere 4 specific staining was extended to the neural crest cells migrating from rhombomere 4 to the second branchial arch (Fig. 5F). Rhombomere 4 is the site which shows the highest level of CRABP-I expression in the developing hindbrain of 9.5 days p.c. mouse embryos (Maden *et al.*, 1992). Comparison of the expression pattern of the *lac*Z transgene driven by *Fugu* promoter sequences with that driven by the mouse and chicken promoters shows that the extent of the expresssion pattern decreases with an increase in evolutionary distance between the species from which the promoter was derived and the species in which it was tested, i.e. the mouse.

Discussion

In this paper we present the cloning of the CRABP-I genes from two different species, the chicken and the Japanese pufferfish, *F. rubripes*. The Fugu gene consists of four exons as is the case for CRABP-I from other species, and as indeed appears to be the case for all other members of the intracellular lipid binding protein family (Bass, 1993). The same is expected of the chicken gene, but we have sequenced only the first three exons. The putative intron/exon borders appear conserved and all contain the expected GT splice donor and AG splice acceptor sites (Breathnach and Chambon, 1981). *Fugu* CRABP-I and chicken CRABP-I are 86% and 94% homologous to the mouse CRABP-I protein respectively. Comparison of chicken with *Fugu* CRABP-I reveals a 85% homology over the first three exons. Taken together with the 100% homology between bovine and mouse CRABP-I, and the 99.3% homology with human CRABP-I, these data show the extremely high degree of conservation of the CRABP-I gene (Fig. 6). This indicates that there has been a strong selective pressure on the gene through evolution. Interestingly this selective pressure has

```
                    1
Mouse CRABP-I       MPNFAGTWKM RSSENFDELL KALGVNAMLR KVAVAAASKP HVEIRQDGDQ FYIKTSTTVR TTEINFKVGE
Rat CRABP-I         .......... .......... .......... .......... .......... .......... ..........
Bovine CRABP-I      .......... .......... .......... .......... .......... .......... ..........
Human CRABP-I       .......... .......... .......... .......... .......... .......... ..........
Chicken CRABP-I     .....R.... .......... .......... .......... .......... .......... .......I..
Fugu CRABP-I        .......... K......... ......T... ........N. ........EK .......... ......HI..
Ambystoma CRABP-I                                                    .....R .......... ......T...

                    71
Mouse CRABP-I       GFEEETVDGR KCRSLPTWEN ENKIHCTQTL LEGDGPKTYW TRELANDELI LTFGADDVVC TRIYVRE
Rat CRABP-I         .......... .......... .......... .......... .......... .......... .......
Bovine CRABP-I      .......... .......... .......... .......... .......... .......... .......
Human CRABP-I       .......... .....A.... .......... .......... .......... .......... .......
Chicken CRABP-I     S......... .....A.... ....Y.K... I......... .......... .
Fugu CRABP-I        E.D....... ..K......S ....R.K... V........F. ....NG...T .V..... .......
Ambystoma CRABP     E.N....... ..K..A.... ....Y.K... V..T...... ......E... .......
```

Fig. 6. Sequence alignment of CRABP-I proteins from various species. The deduced amino acid sequences of CRABP-I from chicken and the puffer fish are compared to the known sequences from mouse, rat, cow, axolotl (*Ambystoma*) and human CRABP-I. Mouse CRABP-I is used as the paradigm. Dots indicate identical residues, and amino acid differences are shown.

not been confined to the RA binding pocket of CRABP-I but includes the whole protein. This implies that the configuration of the ligand binding pocket is critically dependent on the exact structure of the whole protein, and that a change elsewhere in the protein structure would alter the RA binding capacity significantly, perhaps lowering the affinity for RA below that of the RA receptors. Alternatively CRABP-I may interact with other proteins that require its specific structure for recognition, as has been suggested previously (Donovan *et al.*, 1995). These interacting proteins could be enzymes involved in RA metabolism or may be the nuclear RA receptors. The presence of CRABP-I in the nucleus of certain cell types has been reported recently, suggesting the possibility of a direct transfer of RA from CRABP-I to the nuclear receptors (Gustafson *et al.*, 1996).

In addition to the coding regions of the genes we have also sequenced the promoter regions of both the chicken and *Fugu* CRABP-I genes, with the aim of identifying conserved elements that could be involved in the transcriptional regulation of the gene. This approach has proven to be fruitful in the study of the regulation of the Hoxb1 and Hoxb4 genes (Marshall *et al.*, 1994; Aparicio *et al.*, 1995; Morrison *et al.*, 1995; Popperl *et al.*, 1995). We have investigated the transcriptional regulation of the CRABP-I gene during mouse development (Kleinjan, *et al.*, 1997), and found that CRABP-I expression in the developing central nervous system of the mouse is regulated via specific elements located within the 3 kb upstream region of the gene. A similar study (Means *et al.*, 1997) hypothesized the presence of another regulatory element further upstream, but this assumption was based on the absence of expression in a very small number of transgenic embryos. By extensive deletional analysis of the 3 kb upstream region we have clearly shown that the elements in this region are sufficient to direct expression in the CNS of the mouse embryo. Surprisingly, we are unable to recognize these elements in the 3 kb upstream sequences of either the chicken or *Fugu* genes. This may be due to the fact that the recognition sequences of the transcription factor binding sites have diverged from the consensus sites such that they are no longer recognizable. The expression patterns of mouse CRABP-I and chicken CRABP-I have been well studied (Maden *et al.*, 1989; Vaessen *et al.*, 1990; Ruberte *et al.*, 1991; Maden *et al.*, 1992; Ruberte *et al.*, 1992), and their expression patterns during development are largely identical. However, small differences between the two species exist, for instance in the developing hindbrain region. After the appearance of rhombomeres (rh) in the hindbrain, CRABP-I expression in the mouse is found at low levels in rh2, at high levels in rh4–6, and at decreasing levels again caudally (Maden *et al.*, 1992; Leonard *et al.*, 1995). In the chicken, CRABP-I is detected at high levels in rh4, at lower levels in rh5, and at high levels again in rh6, with a subsequent decrease more caudally (Maden *et al.*, 1990). At present the expression pattern of CRABP-I in *Fugu* embryos is not known. We have made transgenic mice with the upstream regions of chicken and *Fugu* CRABP-I driving a *lacZ* reporter gene, and compared these to the expression patterns generated from either a *lacZ* or an epitope tagged reporter gene driven by the equivalent mouse promoter region. In three independent transgenic embryos with the chicken promoter construct we find expression of the *lacZ* reporter gene in the region of the hindbrain that covers rh2 and rh4–6 (Fig. 6B). The restricted expression of *lacZ* in the hindbrain region suggests that the chicken CRABP-I upstream and promoter sequences, when introduced into transgenic mice, drive an expression pattern that only partly recapitulates the endogenous mouse expression pattern. When the promoter region from *Fugu* CRABP-I was tested, the transgenic embryos showed specific and reproducible expression of *lacZ* only in rh4. Thus the domain of *lacZ* expression driven by the promoter region of *Fugu* CRABP-I is an even smaller subdomain of the mouse expression domain than that driven by the chicken CRABP-I promoter region.

Thus, the regulatory regions of the gene have been much less conserved than the coding sequence. The reporter construct containing the *Fugu* upstream region shows that the regulatory mechanisms driving CRABP-I expression have been best conserved in rh4, suggesting that rh4 may be the most important site of CRABP-I expression. Rh4 lies at the boundary between the highly RA sensitive anterior hindbrain and the less RA sensitive posterior hindbrain. RA treatment of early stage embryos results either in complete loss or in a posteriorisation of

anterior hindbrain structures. Typically, these effects are limited to the hindbrain anterior of rh4. A re-specification of the identity of rh2 into a rh4 identity is seen in mouse embryos treated with RA at day 7.5 p.c. (Marshall *et al.*, 1992). Interestingly, the restricted expression of the Hoxb1 gene in rh4 is controlled by RA. The expression of Hoxb1 in rh4 is set up by a positive enhancer that generates expression of Hoxb1 which also extends into rh3 and 5. A repressor containing two RA response elements negatively regulates the expression in rh3 and 5 and restricts Hoxb1 expression specifically to rh4 (Studer *et al.*, 1994). This rhombomere specific activation and repression of Hoxb1 can not be accounted for by a specific distribution of the RARs/RXRs in the hindbrain. CRABP-I is thought to be involved in the regulation of free RA levels. It is well possible that the expression of CRABP-I is specifically important in rh4, where it has to keep the RA that is available for the RARs below a certain threshold level, and that therefore its regulatory mechanisms have been best conserved in that particular rhombomere.

The evolutionary paths of mice and teleost fish, like *Fugu*, have diverged approximately 430 million years ago, while the paths of chicken and mice have diverged 200 million years ago. Thus with increasing evolutionary distance between species an increasing loss of transspecies regulatory potential on the CRABP-I gene occurs. A more detailed analysis of this phenomenon will require the generation of transgenic lines with larger constructs, and the incorporation of more species into the analysis. In this respect it would be very interesting to find out whether amphioxus (*Brachiostoma floridae*), the closest living invertebrate relative of the vertebrates, has a CRABP gene, and whether the promoter region of that gene has any regulatory potential in transgenic mice. Although amphioxus lacks apparent brain structures it has been shown recently that amphioxus embryos treated with RA show patterning defects of their anterior nervous system similar to those seen in vertebrates (Holland and Holland, 1996).

In summary, we have isolated and sequenced the genomic loci of CRABP-I from the chicken and pufferfish, and confirmed that CRABP-I has been strongly conserved through evolution. The promoter regions from the two species directed the expression of a *lacZ* reporter gene in transgenic mice to specific subsets of the murine expression domain. A larger part of the murine expression pattern was reproduced by the chicken promoter region than by the pufferfish, reflecting the relative evolutionary distance between the organisms.

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ti n f additional genetic influences inherited from her father's (IV-17) branch of the family, might contribute to her susceptibility to bilateral breast cancer. In fact, other incidences of cancer have been reported in the pedigree of IV-17 (ref. 12). More extensive sequencing of the *p53* gene, encompassing areas beyond the four hot-spot regions, should provide further clarification.

It has been suggested that point mutations in the conserved region of the *p53* gene can cause conformational changes in the encoded nuclear phosphoprotein. Mutant p53 protein forms a complex with wild-type p53, thereby inhibiting the normal functions of the latter[17,18]. Furthermore, mutations in wild-type p53 protein can abolish the transformation-suppressing activity of the normal p53 in a *ras* complementation assay[19]. The addition of wild-type *p53* complementary DNA can suppress the growth of human glioblastoma[20] and colorectal carcinoma[21] cells. Although the precise functions of the p53 protein remain to be elucidated, several studies of the interaction between p53 protein and viral oncoproteins such as the simian virus 40 T antigen[22,23], adenovirus E1b[24], and papilloma virus E6[25], or studies of the functions of p53 involving the microinjection of anti-p53 antibody[26], indicate that p53 may be essential for the control of cell proliferation. Because affected individuals of the family we studied often have more than one type of neoplasm, a defect in a gene such as *p53*, which is expressed in a variety of cell types, might disadvantage cells of these individuals in terms of the control of normal cell proliferation. Additionally, this mutation in *p53* was found only in NSFs of affected individuals.

Comparisons between normal and tumour tissue from patients with non-hereditary colorectal[7] or lung cancer[8], have demonstrated that neither allelic loss nor *p53* mutations occur in these patients' unaffected cells. The inherited nature of the *p53* mutation that we have now identified in noncancerous cells may represent an early genetic alteration having a predisposing effect in carcinogenesis in the family we studied.

Note added in proof: While this paper was being accepted for publication, similar findings in Li-Fraumeni syndrome were reported[27]. □

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Site-independ nt expression of the chicken β^A-globin g ne in transgenic mice

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THE level of expression of exogenous genes carried by transgenic mice typically varies from mouse to mouse and can be quite low. This behaviour is attributed to the influence of the mouse chromatin near the site of transgene integration[1,2]. This 'position effect' has been seen in transgenic mice carrying the human β-globin gene. It was however, abolished when DNase I hypersensitive sites (normally found 65 to 44 kilobases (kb) upstream) were linked to the human β-globin transgene[3-5]. Thus, the upstream DNA (previously named a dominant control or locus activation region, now denoted a locus control region) conferred the ability to express human β-globin at high levels dependent on copy number on every mouse carrying the construct. We report here an investigation of chicken β^A-globin gene expression in transgenic mice. A 4.5-kb fragment carrying the β^A-globin gene and its downstream enhancer[6-9], without any far upstream elements, is sufficient to ensure that every transgenic mouse expresses chicken globin messenger RNA at levels proportional to the transgene copy number. Thus the chicken DNA elements that allow position-independent expression can function in mice. In marked contrast to the human β cluster, these elements are no farther than 2 kb from the gene. The location of the elements within the cluster demonstrates that position independence can be mediated by DNA that does not define a gene cluster boundary.

To examine the elements contributing to tissue-specific and integration site-independent expression, we produced transgenic mice containing the chicken β^A-globin gene with (β^AE) or without ($\beta^A\Delta$E) the downstream enhancer (Fig. 1*a*). Six of 33 mice developing from embryos injected with β^AE, and 5 of 62 mice developing from $\beta^A\Delta$E-injected embryos carried the transgene. All transgenic mice carried intact β^A-globin genes, integrated in head-to-tail tandem arrays (Fig. 1*b*, and data not shown). Breeding resulted in the establishment of five β^AE lines (one founder did not breed) and six $\beta^A\Delta$E lines (one founder was not bred, and another contained three independently segregating integration stites).

Transgene expression was measured by primer extension. Correctly initiated β^A mRNA was detected in RNA isolated from blood of all six β^AE lines, but not in non-transgenic mice, nor in any of the $\beta^A\Delta$E mice (summarized in Table 1). β^A-globin RNA present at 0.02% of the mouse β^{maj}-globin RNA abundance would have been detected. Pretreatment of the mice with phenylhydrazine[10] increased the total RNA yield 8- to 12-fold (to ~1 $\mu g\ \mu l^{-1}$ blood), but did not affect the relative expression of the mouse α, mouse β^{maj}, and chicken β^A genes. β^A-globin RNA expression was erythroid-specific (present at high levels in blood and spleen; Fig. 2*a*). We presume that the β^A RNA in non-erythroid tissues is due to blood contamination as the $\beta^A:\alpha:\beta^{maj}$ ratio was constant (Fig. 2*a*).

We were curious as to what developmental pattern β^A would exhibit in the mouse. In the chicken, the β^A gene is first expressed in yolk sac in mid-stage embryos and is the predominant β-globin thereafter[11]. In both transgenic mouse lines tested (Fig. 2*b*), the β^A transgene is expressed in 11.5-day embryos (when all of the embryonically derived erythroid cells are produced in

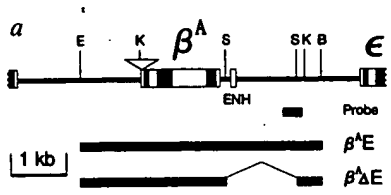
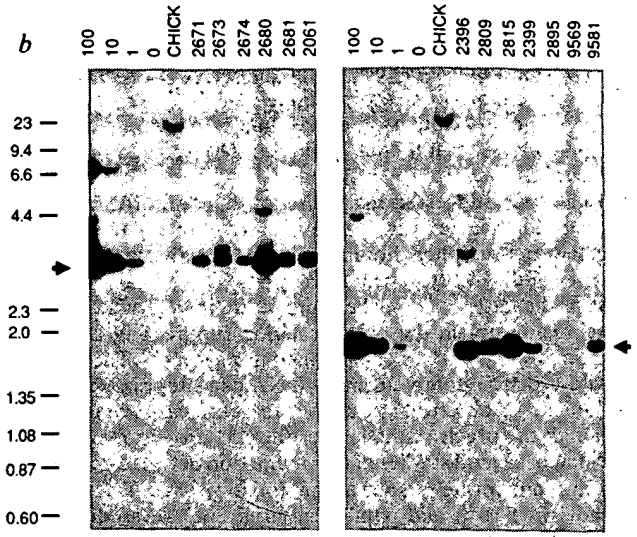


FIG. 1 Production of transgenic mice. *a*, Map of the chicken β-globin locus. The β^A and ε genes (transcribed from left to right), β^A/ε enhancer (ENH), DNA fragments (β^AE, $\beta^A\Delta$E) used for making transgenics, and hybridization probe are indicated. β^AE is a 4.45-kb fragment extending from 1.05 kb upstream of the β^A transcription start site to 0.94 kb 5′ of the ε start site. It contains a 35-base pair (bp) insert with a new *Kpn*I site in the 5′, transcribed, untranslated region of the β^A gene[26]. $\beta^A\Delta$E is the same as β^AE, except for a 1.25-kb *Sac*I deletion that begins ~90 bp 3′ of the polyadenylation site and removes the β^A/ε enhancer. The probe, I142, is a 362-bp *Bgl*II/*Kpn*I fragment. Restriction sites are: E, *Eco*RI; K, *Kpn*I; S, *Sac*I; and B, *Bam*HI. *b*, Southern blots of DNA from transgenic mice. Transgenic FVB/N mice were produced by injection of DNA[27], their tail DNA was digested with *Kpn*I, and subjected to Southern blotting. Left panel, β^AE founder animals (except for 2061 which is derived from 2702). Right panel, $\beta^A\Delta$E founder animals and two 2396-derived progeny (2809, carrying integration site A, and 2815 carrying sites A and C). Arrows show the fragments expected from intact, unrearranged transgenes. Fragments smaller than the arrows are derived from rearranged copies of the transgenes. The 4.5-kb (left panel) and 3.2-kb (right panel) bands are the products expected from partial digestion of head-to-tail tandemly integrated transgenes. Non-transgenic mouse DNA augmented with plasmids containing β^AE (left panel) or



$\beta^A\Delta$E (right panel) at the indicated copy number (per diploid genome) is in lanes marked 0, 1, 10, and 100 (bands larger than those indicated by the arrows are partially digested plasmids). Chicken genomic DNA is also shown at a loading corresponding to a copy number of four; the hybridizing band is of the appropriate size as genomic DNA does not carry the inserted *Kpn*I site. The positions of size markers, in kilobases, are at the left.

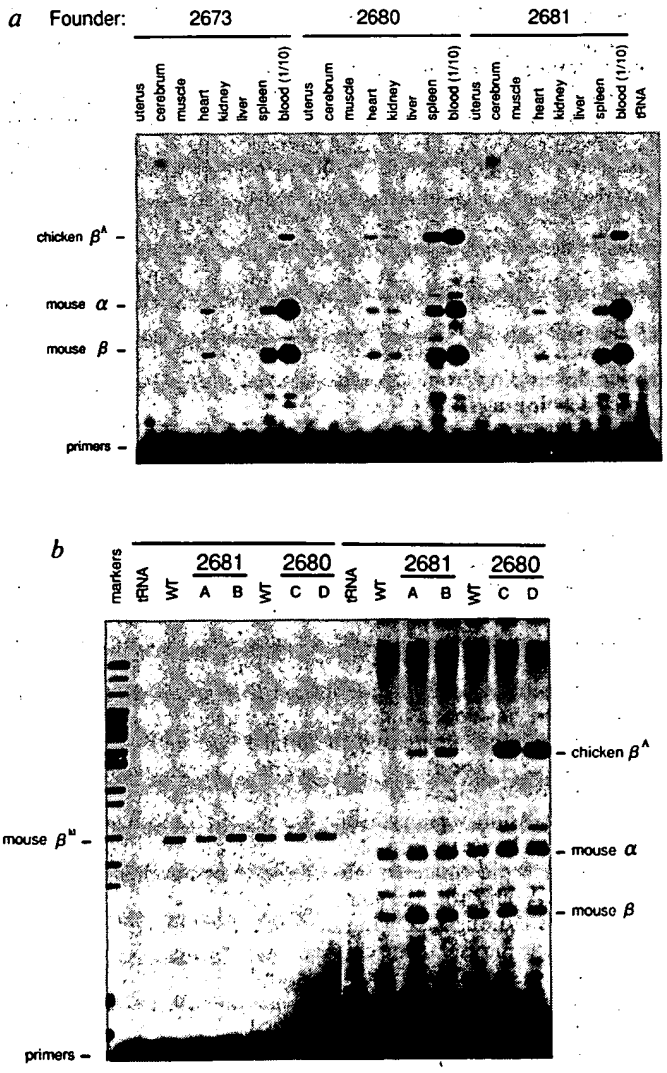


FIG. 2 Tissue and developmental specificity of transgene expression. *a*, RNA was isolated from the indicated tissues of adult female mice (derived from the indicated founders) and measured by primer extension. The chicken β^A, mouse α, and mouse β^{maj} products are so indicated. *b*, Primer extension using total RNA from 11.5-day-old embryos (including placenta and membranes; at 11.5 days all the embryonically produced blood is derived from the yolk sac[12]). The left lanes show primer extensions using the mouse embryonic β^{h1} primer with, from left to right: tRNA, a negative control template; three litter mates, one non-transgenic (WT) and two transgenic (A and B) from breeding a 2681-derived male with a non-transgenic female; and three litter mates, one non-transgenic (WT) and two transgenic (C and D) from breeding a 2680-derived male with a non-transgenic female. The right lanes use the chicken β^A, mouse α, and mouse β^{maj} primers with the same RNAs. Measurement of β^{h1} RNA allows estimation of the contribution of the embryonic erythroid tissue to the total RNA isolated.

METHODS. RNA was isolated[28], primer extension[13] performed with 10 μg (1 μg for blood) of RNA, and the products separated on denaturing 8% polyacrylamide gels. The primers and product sizes are: chicken β^A, 5′-GGTGATGAGCTGCTTCTCCTC-3, 115 nucleotides; mouse α, 5′-CAGGCAGCCT-TGATGTTGCTT-3′, 65 nucleotides; mouse β^{maj}, 5′-TGATGTCTGTTTCTGGGGT-TGTG-3′, 53 nucleotides; and mouse β^{h1}, 5′-ATAGCTGCCTTCTCCTCAGCT-3′, 87 nucleotides. the primers are specific for the indicated mRNAs, except that the β^{h1} primer also detects β^A mRNA. The chicken primer detects the other chicken β-like globins, none of which is present in mice. Assays using a single primer gave identical results to those using multiple primers.

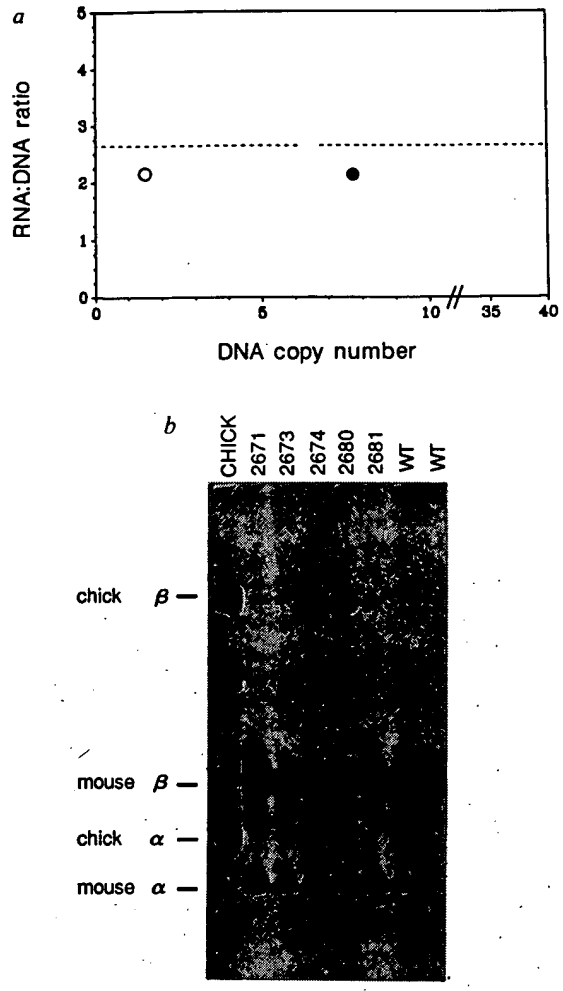


FIG. 3 Copy number dependence of RNA expression. *a*, β^A RNA abundance divided by DNA copy number plotted against DNA copy number. Solid circles represent the mean progeny data and the open circle is the datum for founder mouse 2674 (data from Table 1). The RNA:DNA ratio is the amount of transgene RNA, per transgene copy, expressed as a percentage of the amount of mouse β^{maj} RNA, per gene copy (assuming equally efficient priming and reverse transcription of the different primers and 2 β^{maj} genes per diploid genome). The β^A mRNA abundance per copy averages 2.7% that of mouse β^{maj}. *b*, Triton/acid/urea gel electrophoresis of erythrocyte lysates. A Coomassie-stained gel with lysates from five founder mice, two non-transgenic (WT) mice, and an adult chicken (CHICK) is shown. Positions of the various globins are as indicated. Lysate preparation and electrophoresis were essentially as described[29]. The FVB/N strain used here carries the 'diffuse' (*Hbb*^{d}) allele (C. Hansen, personal communication).

TABLE 1 Transgene DNA copy number and RNA abundance

Line	Founder DNA copy number	Founder RNA abundance	Progeny DNA copy number	Progeny RNA abundance
β^AE				
2671	1.7	2.1	3.2±0.5 (3)	3.1±0.2 (3)
2673	2.2	6.3	6.5±1.0 (11)	8.3±1.6 (6)
2674	1.5	1.6		
2680	30		37±7.6 (5)	61±21 (4)
2681	5.7	7.1	7.2±0.1 (2)	11.9±0.7 (4)
2702	1.8	1.4	7.7±0.5 (2)	8.3±2.1 (3)
$\beta^A\Delta$E				
2396(A+B+C)	72	<0.2		
2396(A)			28±12 (6)	<0.02 (3)
2396(B)			46±17 (3)	<0.02 (1)
2396(C)			63±7 (2)	<0.02 (2)
2399	10	<0.02	9.8±0.1 (3)	<0.02 (2)
2404	0.3	<0.02	0.9±0.1 (7)	<0.02 (3)
9569	1	<0.02		
9581	11	<0.02	24±10 (2)	<0.02 (2)

DNA copy number is presented in copies per diploid genome. β^A RNA abundance is expressed as percentage of mouse β^{maj} RNA, uncorrected for either β^A or β^{maj} gene copy number. Numbers are mean ±s.d. (number of independent observations). Independent observations are defined as different animals, except for pairs of RNA measurements on the same animals bled at different times. Founder 2396 had three separate integration sites, denoted A, B, and C.

DNA copy number was measured by slot blotting[24]. Tail DNA (3 μg) was bound to GeneScreen Plus, in duplicate, and detected by hybridization with probe I142. Linearly exposed autoradiograms were scanned and copy number was calculated from standards on the same blot by linear interpolation. Correct slot loading was confirmed by rehybridizing the blot to a glyceraldehyde 3-phosphate dehydrogenase cDNA probe[25]. The actual copy number is integral in the progeny mice without any rearrangements. RNA abundance in blood was measured by primer extension (Fig. 2). The β^A, α, and β^{maj} bands were scanned, and integrated, and β^A was normalized to the mouse globins in the same sample. From the specific activities of the primers, β^A RNA abundance as a percentage of mouse β^{maj} RNA was calculated.

the yolk sac)[12]. Thus in the mouse, β^A expression begins in the yolk sac and continues through adulthood (Fig. 2*b* and data not shown).

To determine whether locus control region (LCR) activity was present in the β^A fragment, we measured the transgene DNA copy number and RNA abundance (Table 1). Mouse α-globin mRNA served as an internal control for measurement of β^A RNA, confirming equal amounts of erythroid RNA in each sample. The constant mouse β^{maj} message levels demonstrated that transgene expression does not affect the transcription f the endogenous mouse β^{maj} gene. The copy number data suggested that the founder mice were mosaic, and this was supported by the percentage f progeny that were transgenic (25, 28, 18, 28 and 20% f r the β^AE lines). Measurement of the DNA copy number and RNA abundance in progeny mice eliminated the confounding effects of mosaicism. Figure 3*a* shows a plot of β^A RNA abundance per copy number against copy number. It is striking that over a 25-fold range of copy number, the RNA:DNA ratio is constant, exhibiting less than twofold variation.

These data provide evidence that transgene expression is position-independent. Proof of position independence requires only that all lines carrying the same number of gene copies express the same amount of mRNA. That the abundance of β^A RNA per copy number is the same over a 25-fold range of copy number suggests in addition that all copies of the integrated genes are expressed equally. We can compare the coefficient of variation (CV) of the RNA:DNA ratios shown in Fig. 3*a* with that obtained for the human β-blobin gene: the CV is 23% for the chicken gene, comparable to the value of 12% for the human β-globin gene with upstream elements. In contrast, the CV is 130% without these elements (calculated respectively from ref. 5 and ref. 13, −815hβG construct).

The relatively low level of β^A RNA (2.7% that of mouse β^{maj}, per copy) is perhaps not surprising, as far upstream hypersensitive sites[14] (which include enhancers (unpublished observations, see ref. 15)) which might augment the local elements, were not included in the β^AE fragment. It is also possible that in the 250 million years since the common ancestor of chickens and mice, divergence of *cis* elements or *trans* factors may have occurred (see ref. 16 for an example), resulting in less efficient expression of the heterologous gene. Dissociation of

position independence from high level expression also occurs in the human β-globin locus[17]. Irrespective of the level of β^A RNA, it is clear that the chicken elements mediating position-independent expression can function in mice. Thus the mechanisms and mediators responsible for expression independent of integration site arose before the divergence of Mammalia and Aves and have not changed significantly since then. The β^AE transgenic mice also express β^A-globin protein in a copy-number dependent manner (Fig. 3*b*).

This strict dependence on copy number, together with the observation that every transgenic mouse expresses the chicken β^A-globin gene, is evidence that the β^AE fragment carries the information necessary for position independence. We do not know which regions within β^AE cause position independence, but the β^A/ε enhancer is nuclease-hypersensitive in chromatin and thus a likely candidate. It is interesting that both the chicken β^AE fragment and complete constructs of the human β-globin LCR contain the strongest enhancer of their respective clusters[15,18]. Both the chicken β^AE and the larger, more effective versions of the human β LCR constructions contain matrix attachment regions (putative sites of DNA attachment to the nuclear matrix or scaffold)[19,20]. The functional contribution of matrix attachment regions to LCR activity is, however, unclear.

The chicken globin element we describe is located in the midst of the gene cluster, whereas the elements of the human β-globin LCR are far upstream from the genes they regulate[5,15,21-23]. The intracluster location demonstrates that position independence is not necessarily mediated by regions at the boundary of a gene cluster or chromatin domain.

Little is known about the mechanisms bestowing position-independent gene expression. For example, there is no evidence that the elements mediating this effect share a unique, defining sequence motif or bind a distinct protein. The ability of a relatively well understood part of the chicken β-globin locus to confer position independence bodes well for our ability to dissect the details of this phenomenon. □

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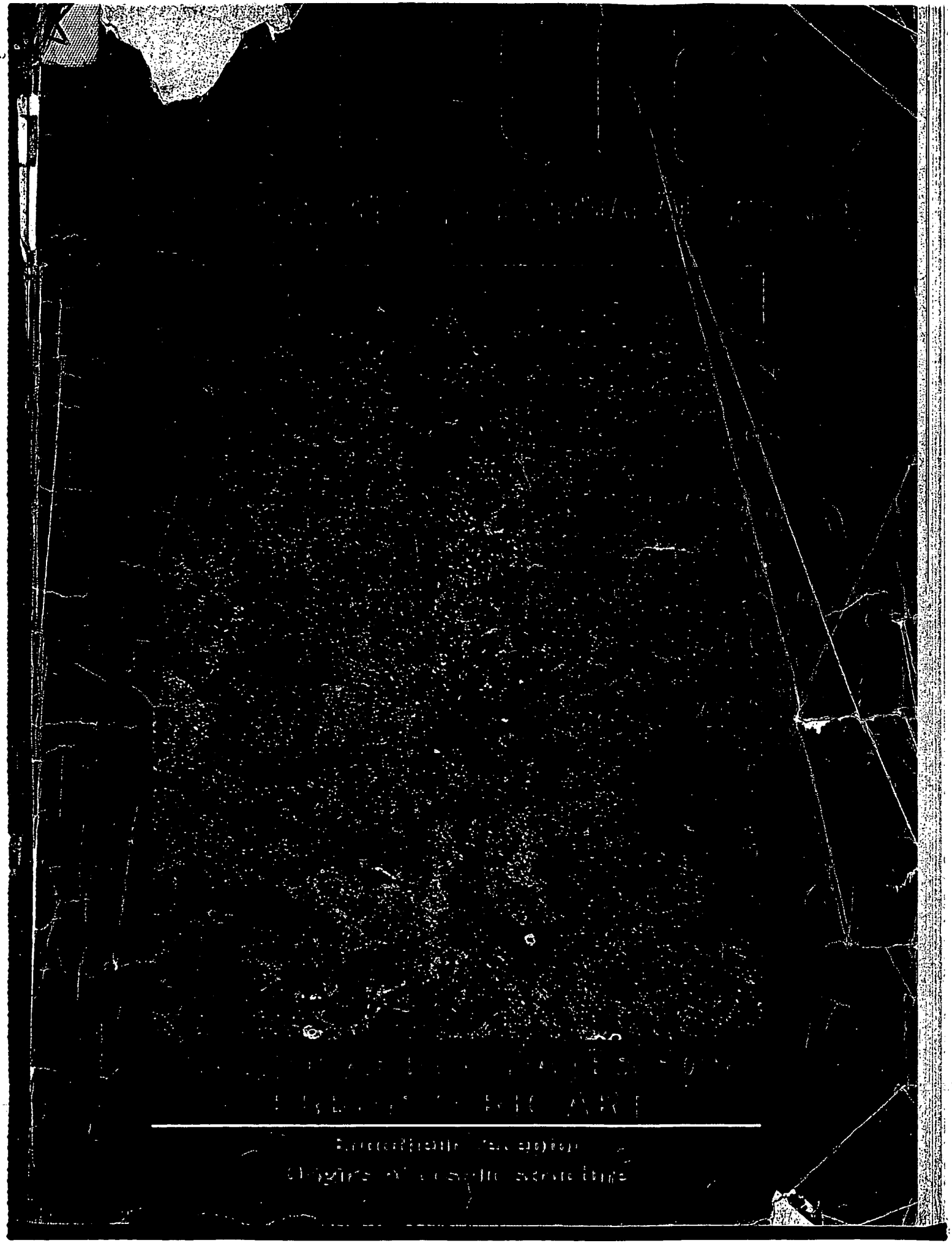

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Association of the 5′HS4 sequence of the chicken β-globin locus control region with human EF1α gene promoter induces ubiquitous and high expression of human CD55 and CD59 cDNAs in transgenic rabbits

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Key words: transgenic rabbits, β-globin 5′HS4, CD55 cDNA, CD59 cDNA, xenotransplantation

Abstract

Whatever its field of application, animal transgenesis aims at a high level of reproducible and stable transgene expression. In the case of xenotransplantation, prevention of hyperacute rejection of grafts of animal origin requires the use of organs expressing human inhibitors of complement activation such as CD55 (DAF) and CD59. Pigs transgenic for these molecules have been produced, but with low and variable levels of expression. In order to improve cDNA expression, a vector containing the 5′HS4 region from the LCR of the chicken β-globin locus and the promoter and the first intron from the human EF1α gene, was used to co-express human CD55 and CD59 cDNAs in transgenic rabbits. The transgenic lines with the 5′HS4 region displayed dramatically enhanced CD55 and CD59 mRNA concentrations in brain, heart, kidney, liver, lung, muscle, spleen and aortic endothelial cells in comparison with the transgenic lines without the 5′HS4 region. In the absence of the 5′HS4 region, only some of the transgenic lines displayed specific mRNAs and at low levels. Human CD55 and CD59 proteins were detectable in mononuclear cells from transgenic rabbits although at a lower level than in human mononuclear cells. On the other hand, primary aortic endothelial cells from a bi-transgenic line were very efficiently protected *in vitro* against human complement-dependent lysis. Transgenic rabbits harbouring the two human inhibitors of complement activation, CD55 and CD59, can therefore be used as new models in xenotransplantation. Moreover, the vector containing the 5′HS4 region from the LCR of the chicken β-globin locus seems appropriate not only for xenotransplantation but also for any other studies involving transgenic animals in which cDNAs have to be expressed at a high level in all cell types.

Introduction

Whatever its field of application, animal transgenesis is intended to result in high level, reproducible and stable expression of transgenes. This technology has recently penetrated new domains of medical research with the production of genetically modified animals as potential organ donors. The choice of the animal donor should determine the type of genetic modifications leading to tolerance of the xenograft. Non-human primates have first been considered because of their phylogenetic closeness to humans. However, ethical issues and infectious risks presently lead the scientific community to rather use animals phylogenetically distant from the human species, such as the pig. This makes the immunological problems more difficult to solve. Indeed, while transplantation of organs from primate donors to humans is followed by an acute cell-

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mediated rejection within 7–10 days, that of porcine organs is followed by a hyperacute rejection (HAR) within minutes or hours (Platt and Bach, 1991). This HAR is initiated by anti-pig preformed antibodies present in the human recipient, that combine with xenoantigens on the surface of graft endothelial cells and provoke the recipient's complement cascade. This phenomenon induces a strong activation of endothelial cells that leads to vasospasm, local thrombosis, disruption of endothelium and finally to destruction of the graft (Platt et al., 1991).

Since complement activation is the initiating event, use of organs from genetically modified pigs which express genes for proteins inhibiting human complement activation, could make it possible to overcome HAR. CD55, also named DAF (Decay Accelerating Factor) (Lublin and Atkinson, 1989), and CD59 (Schönermark et al., 1986) would have complementary effects since they inhibit complement activation at two levels: CD55 has a decay accelerating activity for C3-convertases of both alternative and classical pathways (Lublin and Atkinson, 1989), while CD59 blocks membrane attack complex assembly after the stage of C7 binding (Rollins and Sims, 1990). Transgenic pigs harbouring these two human genes have been obtained by micro-injecting native CD55 and CD59 genomic DNA fragments containing the majority of their regulatory elements (Cozzi and White, 1995; Diamond et al., 1996) or by using constructs including the transcribed parts of the genes and heterologous promoters (Kroshus et al., 1996). However, the level of expression of the transgenes varies from one animal to another and between the different organs of the same animal (Cozzi et al., 1997). Therefore, further research is needed to obtain homogeneous levels of transgene expression and alternative solutions have been proposed. A cDNA is generally easier to use than the whole corresponding genomic DNA fragment but often leads to poor expression of the transgenes (Palmiter et al., 1991; Petitclerc et al., 1995). The yield has been improved by adding a heterologous intron between the promoter and the coding region (Palmiter et al., 1991), some introns being obviously more potent than others (Petitclerc et al., 1995). However, despite the presence of introns, some transgenes containing cDNA may remain silent and many investigators are still trying to improve the expression vectors. On the other hand, it is well known that the expression of a transgene is generally dependent on its site of integration in the host genome. It may be completely repressed especially when integration takes place in the centromere region composed of heterochromatin (Festenstein et al., 1996). Moreover, when a large number of transgene copies is integrated, their expression is often low. This may be explained by the capacity of the transgenes, integrated as multicopies, to interact with each other by forming loops and preventing transcription factors to reach the promoter. Nevertheless, in a certain number of cases, transgenes are highly and faithfully expressed when they are associated with particular regulatory regions. For example, in the human CD2 locus, authors have shown that HS3, the most distal site among DNAse I hypersensitive sites of the upstream regulatory elements (also called Locus Control Region (LCR)), is sufficient to overcome heterochromatin-mediated gene repression (Festenstein et al., 1996). Another example is given by the human β-globin gene LCR which is capable of inducing a high, tissue-specific and transgene copy-number-dependent expression in transgenic mice (Grosveld et al., 1987). More recently, Felsenfeld et al. have studied the chicken β-globin LCR and attributed to the 5′HS4 fragment (one of its four DNAse I hypersensitive sites) the majority of its stimulating activity on transgene expression (Chung et al., 1993; Chung et al., 1997; Pikaart et al., 1998). It is well established that all these LCRs contain DNA sequences which can support a copy-number-dependent expression whatever the chromosomal site of integration. It has been suggested that these regions increase the probability of setting up an open chromatin domain by recruiting additional DNA-binding proteins, which favours euchromatin formation and expression of the transgene in all cells (Festenstein et al., 1996; Pikaart et al., 1998). The aim of our study was to design a construct including a stimulating sequence in order to produce transgenic rabbits expressing high levels of CD55 and CD59 proteins, and which could be used as experimental models for xenotransplantation. The advantage of using rabbits as compared to other species is the ease of grafting rabbit organs as compared with the mouse. Furthermore, it is simpler to produce transgenic rabbits than transgenic rats or pigs. We report here the effect of the 5′HS4 fragment from chicken β-globin LCR (Chung et al., 1993; Chung et al., 1997; Pikaart et al., 1998) associated with the ubiquitous promoter from the EF1α human gene on the expression of human CD55 and CD59 cDNAs in transgenic rabbits.

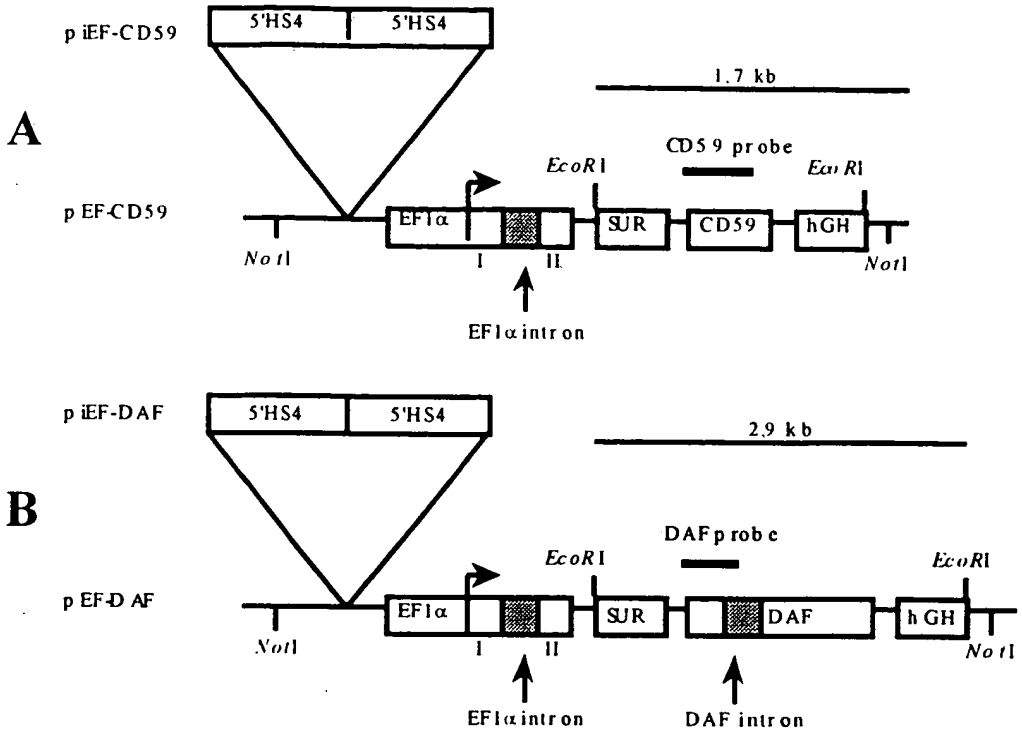

Figure 1. CD55 and CD59 gene constructs with or without the 5′HS4 region from the chicken β-globin LCR. (A) The pEF-CD59 and piEF-CD59 constructs contained the promoter, the first exon, the first intron and the beginning of the second exon from the human EF1α gene, the SUR expression activator, the CD59 cDNA and the human growth hormone gene terminator. The piEF-CD59 contained two copies of the 5′HS4 region. (B) The pEF-DAF and piEF-DAF constructs contained the promoter, the first exon, the first intron and the beginning of the second exon from the human EF1α gene, the SUR expression activator, the CD55 cDNA (with its first intron) and the human growth hormone gene terminator. The piEF-DAF contained two copies of the 5′HS4 region. The probes used for the Southern blots and the expected fragments are indicated by the line above the constructs. Introns are indicated by black boxes. The arrow above the EF1α region indicates the transcription start. *Not*I restriction enzyme was used to generate the transgenes from the corresponding plasmids. *Eco*RI restriction enzyme was used for Southern blot analysis. EF1α = Elongation Factor 1α human gene promoter; I = first exon from human EF1α gene; II = beginning of the second exon from human EF1α gene; hGH = human growth hormone gene terminator.

Materials and methods

Plasmid construction

All techniques used for plasmid construction have been described in Sambrook et al. (1989). The human EF1α gene promoter contained in the pEF321-T plasmid (Kim et al., 1990) was kindly provided by Dr S. Sugano. The 2.5-kb fragment upstream of the initiation codon of the human EF1α gene including the EF1α promoter, the first exon, the first intron and the beginning of the second exon just ending before the ATG initiation codon, was subcloned in the pPolyIII plasmid (Lathe et al., 1987). An expression stimulator (SUR) containing the 5′ untranslated regions from SV40 early genes (S) and from HTLV-1 (UR) (Attal et al., 1996), and the human growth hormone gene transcription terminator (the 0.8 kb *Bgl*II-*Eco*RI fragment) were introduced into the pPolyIII plasmid that already contained the human EF1α promoter to obtain the expression vector pEF0. To obtain piEF0, two copies of the 5′HS4 fragment from the chicken β-globin gene locus (kindly provided by Dr G. Felsenfeld) (Chung et al., 1997) were introduced before the EF1α gene promoter of the pEFO expression vector. The 0.5 kb *Bam*HI fragment of human CD59 cDNA and the 1.7 kb *Bss*HII-*Acc*I fragment of human CD55 minigene including CD55 cDNA and the first 0.5-kb intron (both cDNAs kindly provided by Dr J.-P. Soulillou) were both treated by Klenow DNA polymerase and cloned in the pEFO and the piEFO expression vectors previously restricted by *Spe*I, and also treated by Klenow DNA polymerase. These operations have generated the plasmids pEF-CD59, piEF-CD59, pEF-DAF and piEF-DAF (Figure 1).

Stable expression in CHO cells transfected with piEF-DAF and pEF-DAF

All the reagents used for cell culture, transfection and cloning were from Gibco BRL. CHO cells were cultured in p60 dishes (60 mm diameter) in DMEM-F12 medium supplemented by 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin in a humid atmosphere with 5% CO_2. When cells reached 70% of confluency, they were cotransfected with 1 μg of the plasmid of interest (pEF-DAF or piEF-DAF) and with 0.1 μg of the pRSV-Neo plasmid using Lipofectamine™ as transfecting reagent according to supplier instructions. Two days after transfection, cells were distributed into several p100 dishes and clones were selected using 800 μg/ml of G418.

Production of transgenic rabbits

The transgene fragments generated by the *Not*I restriction enzyme (Figure 1) were purified by adsorption on Glassmilk® (Bio101 Inc.) after separation in 1% agarose gel in the presence of ethidium bromide (agarose-ETB gel) and diluted to 2–4 ng/μl in 5 mm Tris–HCl, 0.1 mM EDTA, pH 7.4. Both the DNA solutions containing CD55 or CD59 constructs were microinjected into the male pronucleus of fertilized rabbit eggs collected from superovulated New Zealand females. These eggs were then transferred to pseudopregnant females (Viglietta et al., 1997). All the experiments were conducted according to local institutional guidelines for care and use of laboratory animals.

Identification of transgenic animals

Transgenic animals (founders and progeny) were identified by PCR analysis performed on DNA extracted from a piece of ear as previously described (Attal et al., 1995). The specific primers used were 5′CAAGGCTAAATTCTGCATCC and 3′GATTTTCCTCTGCATTCAGG for the CD55 transgene, or 5′TCTGCCATTCAGGTCATAGC and 3′TG ACTTAGGGATGAAGGCTC for the CD59 transgene. After 25 cycles of amplification (94°C, 1 min; 55°C, 1 min; 72°C, 1 min), the PCR products (555 bp for CD55 and 336 bp for CD59) were simultaneously detected in 1% agarose-ETB gel.

Primary culture of rabbit endothelial cells

All the reagents used for the primary culture of aortic endothelial cells were from Gibco BRL. Endothelial cells were detached from rabbit aorta with 0.5% collagenase A (Boehringer Mannheim) for 15 min at 37°C followed by scraping. They were seeded into p60 dishes (60 mm diameter) in RPMI 1640 and M-199 medium (v/v) supplemented by 10% fetal calf serum, 10% rabbit serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml amphotericin B. The cultures were incubated in a humid atmosphere with 5% CO_2. When they reached confluency, cells were used for RNA extraction and for complement-mediated lysis assay.

Northern blot analysis of human CD55 and CD59 mRNA concentrations in CHO cells and in various organs from transgenic rabbits

Total RNA was extracted from CHO cells and also from various organs (brain, heart, kidney, liver, lung, muscle, spleen) and aortic endothelial cells from transgenic rabbits by the modified guanidine thiocyanate procedure (Puissant and Houdebine, 1990) originally described by Chomczynsky and Sacchi (1987). Northern blots were performed using 20 μg of total RNA (evaluated by UV light measurement at 260 nm) separated by electrophoresis in a 1.5% agarose gel containing 1 M formaldehyde, and 10 mM EDTA with a Phosphate buffer (20 mM sodium phosphate pH 7, 5 mM sodium acetate and 10 mM EDTA) (Puissant et al., 1994). RNA was fragmented by treatment with 50 mM sodium hydroxide and then transferred by capillarity onto a Biohylon-Z+ membrane (Bioprobe) in the presence of 50 mM sodium phosphate, pH 7. Hybridizations were carried out with the respective ^{32}P-radiolabelled cDNA probes (Random Priming in Sambrook et al., 1989) overnight at 65°C, followed by autoradiography as previously described (Puissant et al., 1994).

Evaluation of transgene copy number in transgenic rabbits by Southern blot analysis

Genomic DNA was extracted from progeny liver by Proteinase K (Boehringer Mannheim) procedure as previously described (Hogan et al., 1986). Genomic DNA from transgenic rabbits was digested for 4 h with *Eco*RI restriction enzyme. A single copy of the CD55 and CD59 genes was evaluated using the genomic DNA from human placenta digested under the

same conditions. Southern blots were performed using 10 μg genomic DNA separated by electrophoresis in a 1% agarose-ETB gel. DNA was fragmented by successive treatments (0.25 M hydrochloric acid for 15 min; 0.5 M sodium hydroxide and 1.5 M sodium chloride for 15 min; 0.5 M Tris and 1.5 M sodium chloride pH 7.4 for 15 min) and then transferred by capillarity onto a Biohylon-Z+ membrane (Bioprobe) in the presence of 50 mM sodium phosphate, pH 7. Hybridizations were carried out overnight at 65°C. Probes were either the 368 bp PCR product obtained with the primers 5′ATGACCGTCGCGCGGCCG and 3′CCTCCCTTTCGATTTTGCCAG for CD55 gene, or the entire CD59 cDNA, both ^{32}P-radiolabelled. The expected radiolabelled bands (2.9 kb for CD55 and 1.7 kb for CD59) were revealed by autoradiography.

Detection of human CD55 and CD59 proteins on mononuclear cells by flow-cytometric analysis

Human CD55 and CD59 proteins were detected on the surface of mononuclear cells from transgenic rabbits (DCi.09, CDi.10 and Di.09 lines). Blood (5 ml) was collected in the presence of 5 mM EDTA and mononuclear cells were separated on Ficoll gradient. Mononuclear cells were washed three times in PBS (pH 7.4) and suspended at a concentration of 10^6cells/ml in PBS, with 1% of bovine serum albumin (BSA) and incubated with monoclonal anti-human CD55 or anti-human CD59 antibodies (Tebu, Le Perray en Yvelines, France) (1/50, 1 h, 4°C). After three washes, the cell suspension (10^6 cells/ml) was incubated with fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse F(ab)′2 (Tebu) (1/20, 1 h, 4°C). After three more washes, cells were submitted to flow-cytometric analysis (Facscan, Becton Dickinson, Mountain View, CA). The specificity of each immunodetection step was controlled by incubating cells with a non-relevant mouse antibody (Tebu). Positive controls were performed by incubating human mononuclear cells with mouse monoclonal anti-human CD55 or anti-human CD59 antibodies.

Complement-dependent lysis of endothelial cells from transgenic rabbits by normal human serum

Primary aortic endothelial cells (passages two and three) from a human CD55-transgenic rabbit (line Di.09), a human CD55 and CD59 transgenic rabbit (line CDi.10) and from a nontransgenic littermate, were collected and cultured as described above. Complement-mediated cytotoxicity tests were carried out as previously described (Malassagne et al., 1998). Spontaneous release of ^{51}Cr was subtracted from test values. Percentages of specific lysis were calculated as indicated below:

$$\text{Percentage} = \frac{\text{experimental cpm} - \text{spontaneous release cpm}}{\text{100\% lysis cpm} - \text{spontaneous release cpm}}.$$

Results

Northern blot analysis of human CD55 mRNA concentrations in CHO cells stably transfected with piEF-DAF and pEF-DAF

Total RNA from CHO clones transfected with piEF-CD55 (i.e. containing the 5′HS4 region from chicken β-globin LCR) was analysed by Northern blot. Results were compared with those obtained in CHO clones transfected with pEF-CD55 (i.e. without the 5′HS4 region). No significant difference in the number of expressing clones and in the expression levels could be noticed between cells harbouring the constructs with or without the 5′HS4 region (data not shown). Consequently, the evaluation of the gene constructs was performed by *in vivo* experiments.

Generation of transgenic rabbits harbouring the human CD55 and CD59 transgenes

Microinjection of both CD55 and CD59 constructs without the 5′HS4 region from the chicken β-globin LCR (Figure 1) generated four double-transgenic founders for CD55 and CD59 (DC.10, DC.18, DC.21 and DC.27), two single-transgenic founders for CD55 (DAF.02 and DAF.07) and one single-transgenic founder for CD59 (C59.18). Microinjection of both CD55 and CD59 constructs containing the 5′HS4 region (Figure 1) generated two double-transgenic founders for CD55 and CD59 (DCi.09 and CDi.10) and two single-transgenic founders for CD55 (Di.01 and Di.09). All these founders were crossed with non-transgenic rabbits. Some of the founders transmitted their transgenes with a very low yield indicating significant mosaicism. In the DC.21 founder, we observed that the CD59 transgene was integrated at two different sites generating two distinct lines: a CD55 and CD59 double-transgenic line (which retained the designation DC.21) and a CD59 single-transgenic line (named C.21).

Table 1. Evaluation by Northern blots of mRNA concentration in various organs from transgenic rabbits without the 5′HS4 region from the chicken β-globin LCR.

Transgenic lines	Detected transgene	Organs						
		Brain	Heart	Liver	Muscle	Lung	Spleen	Kidney
DC.10	CD55	++	+++	++	+	±	±	0
	CD59	++	++	+	+	+	+	±
DC.18	CD55	+	+	±	0	0	±	±
	CD59	0	±	±	0	0	±	0
DC.21	CD55	0	0	0	0	0	0	0
	CD59	0	0	0	0	0	0	0
DC.27	CD55	±	0	±	0	±	±	±
	CD59	0	0	0	0	0	0	0
DAF.02	CD55	?	0	0	0	0	0	0
DAF.07	CD55	+	+	0	±	±	0	±
C59.18	CD59	0	0	0	0	0	0	0
C.21	CD59	0	0	0	0	0	0	0

Autoradiographies were of two and three days to detect CD55 (DAF) and CD59 mRNAs respectively.

Northern blot analysis of human CD55 and CD59 mRNA concentrations in various organs from transgenic rabbits

Total RNA was extracted from various organs of the F1 transgenic rabbits (brain, heart, kidney, liver, lung, muscle and spleen) and from the primary aortic endothelial cells when possible. Table 1 shows that, in transgenic rabbits without the 5′HS4 region from the chicken β-globin LCR, only the DC.10 line showed a broad pattern of expression of both transgenes. The mRNAs were present in most of the tested organs at the expected molecular weight of 1.9 kb for the CD55 mRNA and 1.25 kb for the CD59 mRNA. The highest level of expression of both transgenes was found in brain, heart and liver. In the DC.18 line, the expression was quite significant and observed essentially in the same organs as in DC.10 line, although at a lower level. The other lines of transgenic rabbits without the 5′HS4 region expressed the transgenes either weakly (DC.27, DAF.07) or not at all (DC.21, C59.18, DAF.02 and C.21). Figure 2A shows the Northern blots from three transgenic lines without the 5′HS4 region from the chicken β-globin LCR expressing the CD55 transgene (DC.10, DC.18 and DAF.07 lines).

The presence of the 5′HS4 region dramatically increased the number of transgenic rabbits expressing the transgenes (Table 2). Moreover, high concentrations of specific mRNAs were observed in all rabbits harbouring the 5′HS4 region, except in the Di.01 line which did not express the CD55 transgene at all. The transgene expression was no longer restricted to brain, heart or liver but was widespread in all the examined organs. Three transgenic rabbits with the 5′HS4 region

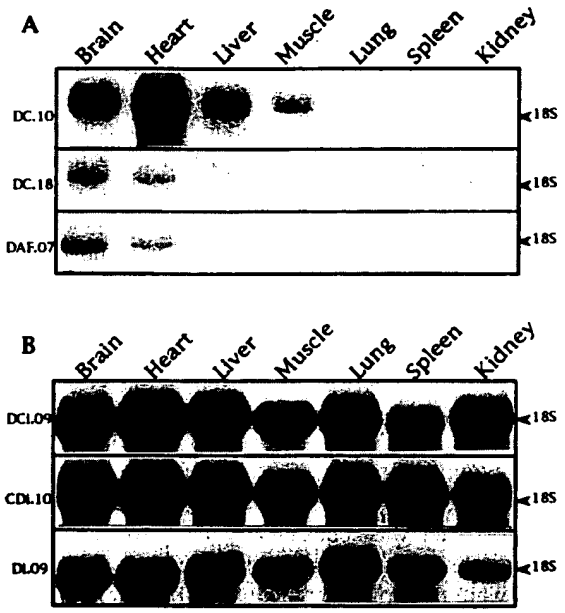

Figure 2. (A) Northern blot analysis of CD55 mRNA in various organs from three transgenic lines (DC.10, DC.18 and DAF.07) not harbouring the 5′HS4 region from the chicken β-globin LCR (two-days autoradiography). (B) Northern blot analysis of CD55 mRNA in organs from three transgenic lines (DCi.09, CDi.10 and Di.09) harbouring the 5′HS4 region from the chicken β-globin LCR (one-day autoradiography).

Table 2. Evaluation by Northern blots of mRNA concentration in various organs from transgenic rabbits with the 5′HS4 region from the chicken β-globin LCR.

Transgenic lines	Detected transgene	Organs						
		Brain	Heart	Liver	Muscle	Lung	Spleen	Kidney
DCi.09	CD55	+++++	++++++	+++++	+++++	++++++	+++++	+++++
	CD59	+++++	++++++	+++++	+++++	++++++	+++++	+++++
CDi.10	CD55	++++++	++++++	+++++	+++++	+++++	+++++	++++
	CD59	++++	++++	++	++	++++	++	++
Di.01	CD55	0	0	0	0	0	0	0
Di.09	CD55	+++++	++++++	++++	+++++	++++	++++++	++++

Autoradiographies were of one day to detect both CD55 and CD59 mRNAs.

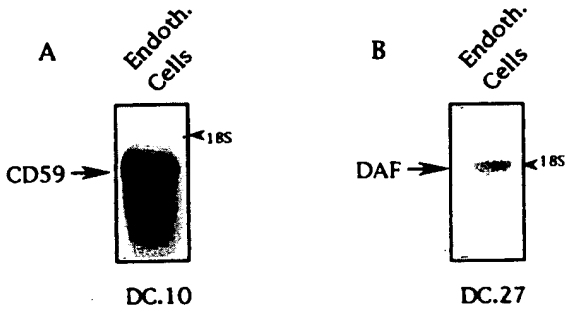

Figure 3. Northern blot analysis of transgene expression in aortic endothelial cells from two transgenic rabbit lines. (A) CD59 mRNA concentration in aortic endothelial cells from DC.10 line (three-day autoradiography). (B) CD55 mRNA concentration in aortic endothelial cells from DC.27 line (two-day autoradiography). 18S RNA position is indicated by the arrows. Endoth. cells = aortic endothelial cells.

expressed the CD55 transgene at a very high level (Figure 2B). Interestingly, the concentration of the mRNAs was high enough to be revealed after no more than a 4-h autoradiography (data not shown). Figure 3 shows that the expression in primary cultures of aortic endothelial cells was effective for the CD59 transgene in the DC.10 line and also for the CD55 transgene in the DC.27 line.

Evaluation of the transgene copy number by Southern blot analysis

CD55 and CD59 copy numbers in transgenic rabbits were evaluated by Southern blot (Figure 4). Table 3 summarizes the CD55 gene copy number of each transgenic line, in comparison with the single copy of human placenta and the corresponding mRNA concentration (Tables 1 and 2). In transgenic rabbits not harbouring the 5′HS4 region (part A of Table 3), CD55 mRNA concentration was at a low level and without any relationship with the corresponding CD55 gene copy number. The DAF.02 and DAF.07 lines which had a high CD55 gene copy number (eight and more than 10 copies, respectively) did not express the transgene, whereas the DC.10 line which had only four CD55 gene copies gave slight expression. In contrast, in the transgenic rabbits harbouring the 5′HS4 region (part B of Table 3), the CD55 mRNA concentration was correlated with the corresponding CD55 gene copy number. In both DCi.09 and CDi.10 lines which had four copies of the CD55 transgene, the mRNA concentration was high, whereas in the Di.09 line with two copies, a lower mRNA concentration was found. In the Di.01 line, the CD55 transgene underwent a recombination that may explain its lack of expression.

Table 4 summarizes the CD59 gene copy number of each transgenic line, in comparison with the single copy of the human placenta and the corresponding mRNA concentration (Tables 1 and 2). In the absence of the 5′HS4 region (part A of Table 4), the mRNA concentration was very low in the animals with four transgene copies (DC.10 and DC.18 lines) and null in those with three copies of the transgene (C59.18 line). It should be noted that the DC.27 line contained a recombined CD59 transgene. In contrast, when the 5′HS4 region was present, a correlation between mRNA concentration and transgene copy number was observed although only two transgenic lines were obtained (part B of Table 4). The DCi.09 and CDi.10 lines, harbouring four and three copies of the CD59 transgene respectively, expressed the transgene at a higher level than the DC.18 or C59.18 lines without the 5′HS4 region and harbouring four and three copies of the transgene, respectively. Moreover, the transgene expression level was higher with four transgene copies (DCi.09 line) than with three transgene copies (CDi.10 line).

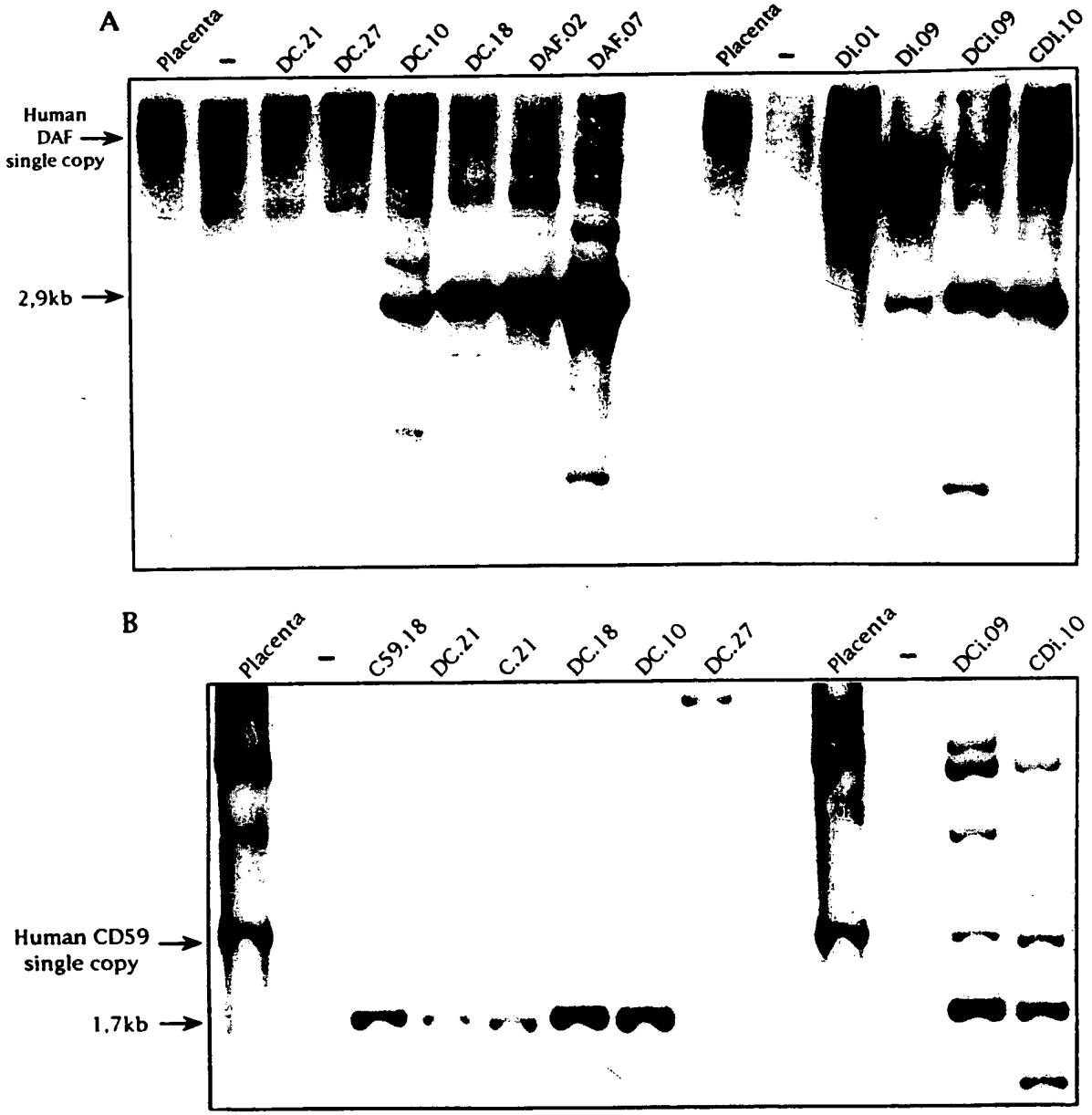

Figure 4. Southern blot analysis of genomic DNA from transgenic rabbits for the evaluation of transgene copy number. (A) Southern blot for the human CD55 transgene. (B) Southern blot for the human CD59 transgene. Each genomic DNA from transgenic rabbits and human placenta (for single-copy control) was digested by *EcoR*I. Probes and restriction enzyme localizations are indicated in Figure 1. The '–' symbol designates a non-transgenic control rabbit.

Table 3. Evaluation of the CD55 (DAF) gene copy number by Southern blot in transgenic rabbits not harbouring (A) or harbouring (B) the 5′HS4 region from the chicken β-globin LCR and comparison with the corresponding mRNA concentration.

Transgenic lines	CD55 transgene copy number[a]	mRNA concentration[b]
A		
DC.10	4	++
DC.18	8	+
DC.21	1	0
DC.27	1	±
DAF.02	8	0
DAF.07	>10	+
B		
DCi.09	4	+++++
CDi.10	4	+++++
Di.01	0	0
Di.09	2	++++

[a]Transgene copy number was evaluated from the Southern blot showed in Figure 4A.
[b]Mean level of mRNA detected in the different tissues of transgenic rabbits (Tables 1 and 2).

Table 4. Evaluation of the CD59 gene copy number by Southern blot in transgenic rabbits not harbouring (A) or harbouring (B) the 5′HS4 region from the chicken β-globin LCR and comparison with the corresponding mRNA concentration.

Transgenic lines	CD59 transgene copy number[a]	mRNA concentration[b]
A		
DC.10	4	+
DC.18	4	±
DC.21	1	0
DC.27	0	0
C59.18	3	0
C.21	2	0
B		
DCi.09	4	+++++
CDi.10	3	+++

[a]Transgene copy number was evaluated from the Southern blot showed in Figure 4B.
[b]Mean level of mRNA detected in the different tissues of transgenic rabbits (Tables 1 and 2).

Detection of human CD55 and CD59 proteins on mononuclear cells by flow-cytometric analysis

As shown in Figure 5, peripheral mononuclear cells from DCi.09, CDi.10 and Di.09 transgenic rabbits, expressed human CD55 or, human CD55 and CD59 proteins at quite significant levels. However, the concentration of CD55 was below that observed in human mononuclear cells.

Cytotoxicity test on aortic endothelial cells from two transgenic rabbit lines

As shown in Figure 6, aortic endothelial cells expressing human CD55 (Di.09) or human CD55 and CD59 proteins (CDi.10) were protected from human complement-mediated cytotoxicity whereas the non-transgenic aortic endothelial cells were not. The maximal rate of lysis of endothelial cells from non-transgenic rabbits reached 46% with human serum at 1/4 dilution. In contrast, the rate of endothelial cell lysis was constantly lower and did not exceed 25% and 10% respectively with the Di.09 and CDi.10 transgenic rabbits.

Discussion

This paper reports the effect of the 5′HS4 region from the chicken β-globin LCR on the expression of human CD55 and CD59 cDNAs in transgenic rabbits, under the control of the ubiquitous promoter from the human polypeptide chain elongation factor 1α gene (EF1α). The aim was to improve an expression vector in order to express cDNAs in all cell types in a reliable manner, and independently of the chromosomal site of integration. The vector described here contains an expression stimulator called SUR which was previously described by Attal et al. (1996). The SUR sequence is able to markedly increase transcription, and allow independent translation of the second cistron in biscistronic mRNAs. Two ubiquitous promoters were tested for their capacity to drive a high expression *in vitro* in transient transfections, the murine phosphoglycerate kinase 1 (PGK1) promoter (McBurney et al., 1994) and the EF1α promoter associated with its first intron (Uetsuki et al., 1989; Kim et al., 1990). The PGK1 promoter is already known to be able to direct *in vivo* expression to an acceptable level (McBurney et al., 1994). Little data on the *in vivo* efficiency of the 2.5 kb EF1α promoter are available (Hanaoka et al., 1991). The EF1α gene is constitutively expressed in most adult tissues and cells with essentially no regulation at the transcriptional level (Uetsuki et al., 1989). This

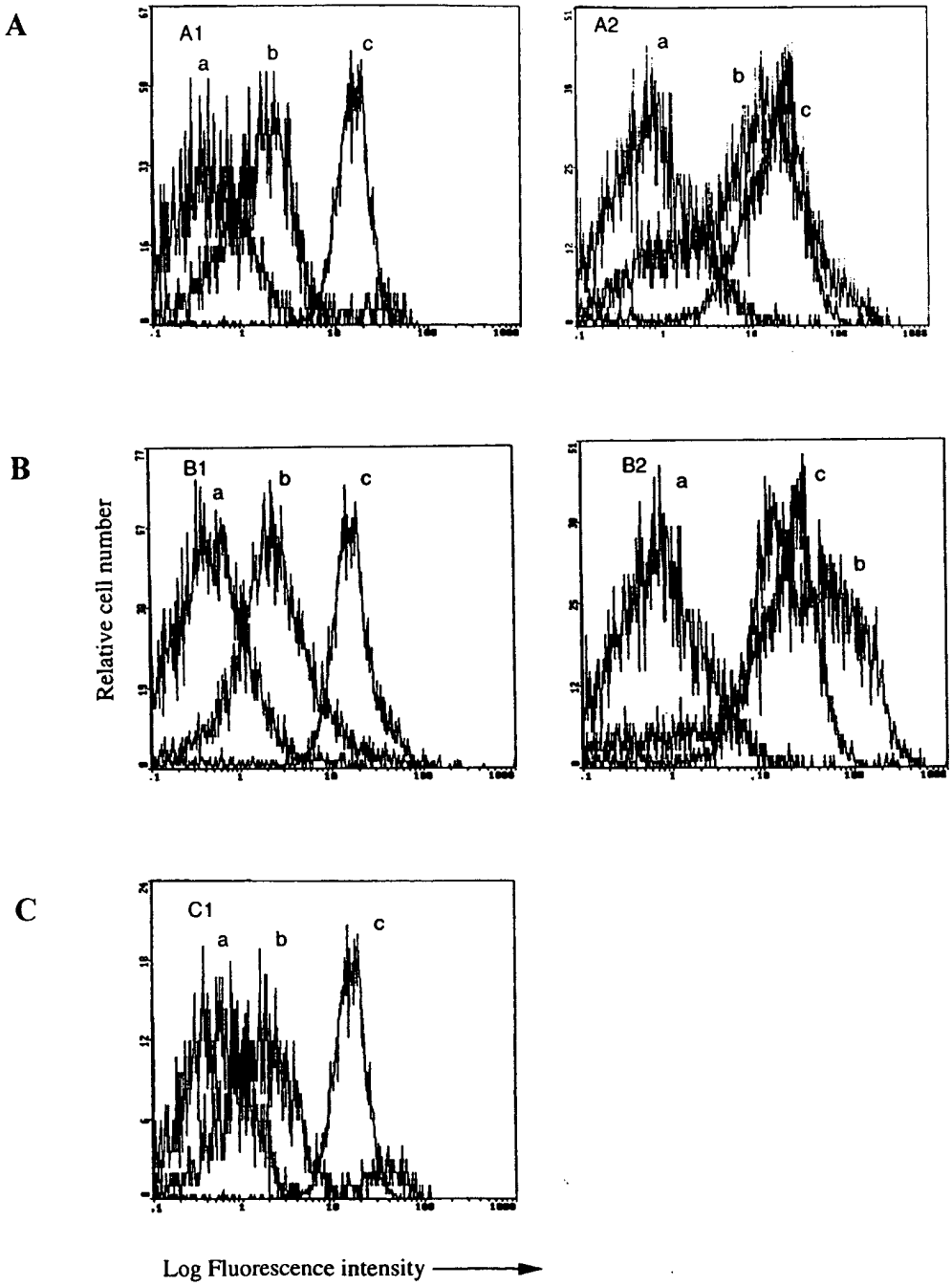

Figure 5. Cell surface expression of hCD55 and hCD59 in transgenic rabbits. (A) Expression of hCD55 (A1) and hCD59 (A2) on mononuclear cells from a negative littermate control (curve a), from a hCD55-CD59-transgenic rabbit (CDi.10 line (curve b)) and from a human donor (curve c). (B) Expression of hCD55 (B1) and hCD59 (B2) on mononuclear cells from a negative littermate control (curve a), from a hCD55-CD59-transgenic rabbit (DCi.09 line (curve b)) and from a human donor (curve c). (C) Expression of hCD55 (C1) on mononuclear cells from a negative littermate control (curve a), from a hCD55-transgenic rabbit (Di.09 line (curve b)) and from a human donor (curve c).

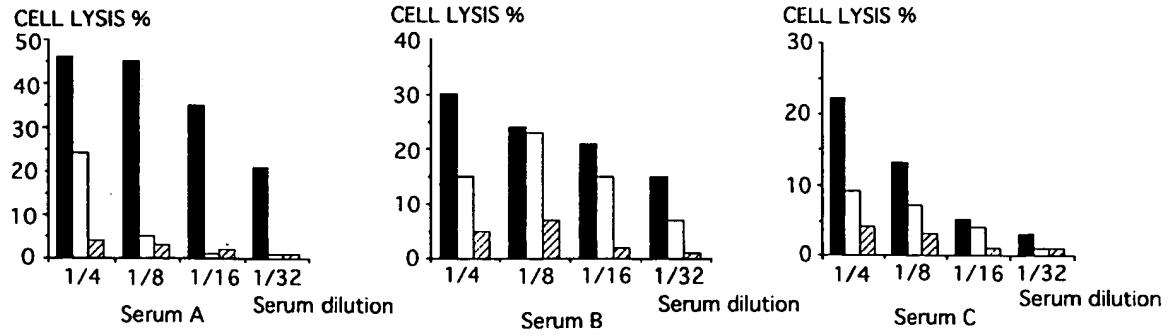

Figure 6. Complement-mediated cytotoxicity assay on rabbit endothelial cells. Rabbit endothelial cells derived from a negative littermate control (solid bars), a hCD55-transgenic rabbit (Di.09 line (open bars)) and a hCD55-CD59-transgenic rabbit (CDi.10 line (hatched bars)). Triplicate data from three separate experiments with three different human sera (A, B and C).

fact was confirmed by the widespread expression of reporter genes driven by the EF1α promoter in most of the tested mammalian cells (Kim et al., 1990) and more efficiently than the RSV or the SV40 early region promoters (Kim et al., 1993). *In vitro*, the 2.5 kb EF1α promoter appeared much more potent than the PGK1 promoter (data not shown). We therefore chose the EF1α promoter to express human CD55 and CD59 genes in rabbits.

The EF1α promoter was able to direct CD55 and CD59 cDNA expression in all the cell types examined, including the aortic endothelial cells from two independent lines (DC.10 and DC.27). This result is important since endothelial cells are the major targets of host complement after blood recirculation in the xenograft. All the transgenic lines harbouring the 5′HS4 region from the chicken β-globin locus expressed the transgenes at a high level. The only silent transgene was rearranged (Di.01 line). Interestingly, the expression was almost the same in all the organs tested, a situation not encountered when the CD55 minigene was used (i.e. with its own promoter) (Cozzi et al., 1997). The 5′HS4 region can therefore be considered as very helpful in association with the EF1α promoter in directing a high-level and ubiquitous expression in all the transgenic lines. Moreover, although few transgenic lines were studied here, the transgene expression seemed to be independent of its integration site and related to the copy number. However, more transgenic lines will be needed to confirm this point. Another recent study also showed that the 5′HS4 region associated with the transthyretin gene promoter allowed the expression of chimeric transactivator GLVP at a high level and in a liver-specific fashion in transgenic mice (Wang et al., 1996). It is presently not clear how the 5′HS4 region is acting. Various studies have suggested that the sequences which allow a satisfactory expression of transgenes prevent the formation of heterochromatin in the transgene region, a phenomenon which leads to an extinction or to a variegated expression of the transgenes (Fraser and Grosveld, 1998). Other studies have attributed to GC-rich regions of the LCRs the role of chromatin-opening activity which makes the transgene accessible to nucleases (Ortiz et al., 1997). In a recent study, Pikaart et al. (1998) observed that the 5′HS4 region prevents the progressive extinction of expression of integrated foreign genes in cultured cells. The action of the 5′HS4 region is accompanied by an increase in the histone acetylation of the integrated DNA. In their work, and also in that published by Wang et al. (1996), two copies of the 5′HS4 region were added on each side of the construct. In our study, the action of the 5′HS4 region was quite effective although it was added only at the 5′P end of the construct. The fact that transgenes are usually arranged in tandem arrays implies that most of the integrated copies were surrounded by the 5′HS4 region.

The presence of human CD55 and CD59 proteins was identified by flow cytometry on the mononuclear cells from the three lines harbouring the 5′HS4 sequence (DCi.09, CDi.10 and Di.09 lines). In these lines, a very high concentration of mRNA was detected. The CD59 concentration was similar to that found in human mononuclear cells. In contrast, the CD55 concentration was lower than in human cells. However, it should be noted that the concentration of CD59 in human mononuclear cells is naturally lower than that of CD55. The concentration of human CD55 and CD59 proteins in the rabbit endothelial cells appeared unexpectedly low in comparison with the very high mRNA level. The human proteins may not be

very stable in rabbit cells. Alternatively, the mRNAs may not be translated at their maximum rate. Indeed, in the EF1α mRNA, the cap region is followed by a short oligoT region (Uetsuki et al., 1989) which is known to reduce translation in quiescent cells (Avni et al., 1997). The vector used in the present study might therefore be improved by removing the oligoT sequence located at the 5'P end of the mRNAs.

It may not be useful to express the genes coding for these inhibitors of complement activation at a level higher than that of the endogenous human genes. Indeed, 70 CD55 molecules per cell were shown to be sufficient *in vitro* to protect endothelial cells from complement-mediated lysis (Medof et al., 1984). Moreover, Cozzi et al. (1997) showed that, beyond 300 CD55 molecules per μm^2 of plasma membrane, no more benefit could be observed in terms of iC3b deposition after complement activation. Van Denderen et al. (1996) also showed that the C3 binding and the cell lysis decreased similarly in heterozygous and homozygous animals despite the two-fold higher level of CD55 gene expression in the latter. The complement-mediated cytotoxicity test showed that both human CD55 and CD59 proteins were functional in transgenic rabbits, since the endothelial cells displayed an almost complete resistance to human complement-mediated lysis. In conclusion, we have described an improved vector for the ubiquitous, high-level, position-independent and possibly copy-dependent expression of cDNAs in transgenic animals. This expression vector containing the EF1α promoter and the 5'HS4 region from the LCR of the chicken β-globin locus seems appropriate not only for xenotransplantation studies but also for any other work involving transgenic animals in which cDNAs have to be expressed at a high level in all cell types. The transgenic rabbits described in the present paper are being studied to evaluate their ability to be used as models for xenotransplantation.

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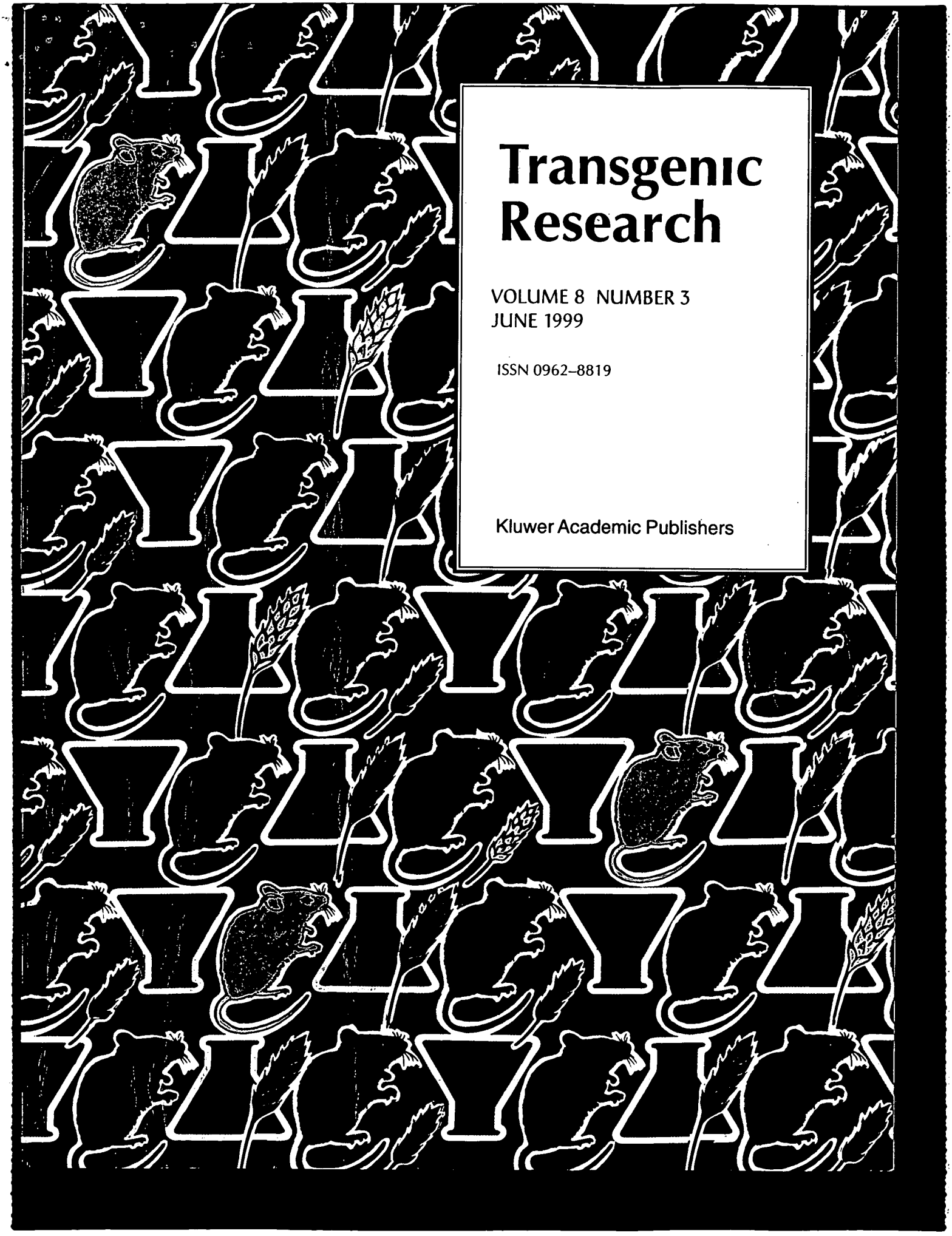

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Distribution of immunoreactive Tamm-Horsfall protein in various species in the vertebrate classes

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Abstract. A sheep antibody to human Tamm-Horsfall protein, the major protein in normal urine, was used in an immunohistological study of organs of 48 species of vertebrate animals, representing the classes Mammalia, Aves, Reptilia, Amphibia, Osteichthyes and Chondrichthyes. Immunoreactivity was shown in the thick limb of the loop of Henle in the kidney of mammals, but there was no reactivity with tissues of birds or reptiles. Superficial layers of the skin of several amphibians and fish, superficial layers of the oral mucosa and gills of fish, and the distal tubules of the kidney of some amphibians, reacted with the antibody. Immunoreactivity with mammalian kidney was removed by passage of the antibody down an immunoadsorption column coated with human Tamm-Horsfall protein, and amphibian immunoreactivity was removed by incubation of the antibody with material prepared from frogs in the same way as Tamm-Horsfall protein. These findings suggest that immunoreactive Tamm-Horsfall protein appeared early in vertebrate phylogeny, initially in skin and gills and later in kidney, and that although conserved in evolution, it shows antigenic differences between amphibians and mammals. Its distribution is consistent with the hypothesis that it acts as a waterproofing agent.

Key words: Tamm-Horsfall protein – Skin – Gills – Kidney – Vertebrata

Introduction

Tamm and Horsfall isolated from human urine a protein that has been shown to be the most abundant protein in urine although its function is still undetermined (Tamm and Horsfall 1950, 1952; Kumar and Muchmore 1990). With an antibody to human Tamm-Horsfall protein, we have previously shown immunoreactivity in tissues of the common frog, *Rana temporaria*, in the distal tubules of the kidney and superficial layers of the epidermis (Howie et al. 1991). This finding suggested (1) that the protein may be an important component of epithelia that absorb sodium and chloride ions but are impermeable to water, and (2) that the protein may have appeared earlier in phylogeny than hitherto realised.

To investigate the phylogeny of Tamm-Horsfall protein, we used the antibody to study organs of various other species, especially skin and kidney.

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Materials and methods

Tissues

Table 1 lists the species and tissues studied. Specimens were taken at necropsy of animals that had been found dead, or from animals shortly after they were killed. Specimens were fixed in either 10% formol-saline or 10% formalin in phosphate-buffered saline, pH 7.4.

Immunohistological method

Fixed tissues were processed into paraffin wax in standard ways and sectioned at about 5 μm thickness. Sections were dried on glass slides, dewaxed in xylene, transferred through decreasing concentrations of alcohol into tap water, and washed. To block activity of endogenous peroxidase, sections were covered with hydrogen peroxide, 2.5% in distilled water, for 5 min, washed, covered with 0.1 M periodic acid in distilled water for 5 min, washed, covered with potassium borohydride, 0.02% in distilled water, for 2 min, and washed again (Heyderman 1979). The IgG fraction of sheep antiserum to human Tamm-Horsfall protein was obtained from The Binding Site Limited, Birmingham, UK. This antiserum had been extensively characterised on human tissues (Howie 1987). It was diluted to 1:600 in phosphate-buffered saline, pH 7.4, with 1% ovalbumin (OA-PBS), and was applied to the sections for 30 min at room temperature on a rocking tray. The sections were rinsed in PBS and washed for 15 min in a bath of PBS with 0.001% Brij 96 detergent (Sigma, Poole, UK) (Heyderman 1979). Donkey antiserum to sheep IgG conjugated to peroxidase (The Binding Site Limited) at 1:100 in OA-PBS, was applied to the sections

Table 1. Species and tissues studied by the immunohistological method for Tamm-Horsfall protein, and kidneys, skin and gills showing positive immunoreactivity; all other tissues were negative. *Notes*: K, kidney; S, skin; G, gills and oral mucosa; 1, liver; 2, intestine; 3, heart; 4, salt gland; 5, eye; 6, spleen; 7, ovary; 8, testis; 9, lung; *, positive immunohistological staining; + severely autolysed

Species name	Common name	Tissues studied
Classes: Chondrichthyes and Osteichthyes		
Acipenser ruthenus	sterlet (freshwater) +	K,S*,G
Arothron nigropunctatus	dog-faced puffer fish (marine) +	K,S*,G
Heteropneustes fossilis	liver catfish (freshwater)	S*
Lepadogaster candollei	common clingfish (marine)	S*
Muraena helena	speckled moray eel (marine) +	K,S*,G
Pangasius sutchi	grass-cutter catfish (freshwater)	K,S*
Polyodon spathula	paddlefish (freshwater) +	K,S*
Salmo trutta	brown trout (freshwater)	K,S*,G*, other (1,2,3)
Scyliorhinus canicula	spotted dogfish (marine)	K,S*,G*, other (1,2,3,4)
Serrasalmus nattereri	red-breasted piranha (freshwater) +	K,S*,G
Spondyliosoma cantharus	Black sea bream (marine)	K, other (1,2,3)
Synodontis sp.	catfish (freshwater) +	K,S, other (5)
Tetradon mbu	puffer fish (freshwater)	K, other (6,7)
Thymallus thymallus	grayling (freshwater) +	S,G
Class: Amphibia		
Amphiuma means	Congo eel or two-toed amphiuma	S*
Typhlonectes sp.	caecilian	K*,S*, other (1,2)
Rana catesbeiana	American bullfrog (×3)	K*,S*, other (1,2,3,8,9)
Rana clamitans	American green frog	S*
Rana limnocheris	rice paddy frog +	K, S*
Rana temporaria	common frog	K*,S*
Colostethus trinitatus	Trinidad poison arrow frog (×2)	K,S*, other (2,5)
Xenopus laevis	African clawed frog (toad)	K*,S*, other (1,2,3)
Rhacophorus leucomystax	bamboo tree frog +	K,S*
Kaloula pulchra	Malayan bullfrog	K*, other (1,3,6)
Hyla versicolor	American grey tree frog +	K, other (1,3)
Ceratophrys cranweli	Columbian horned toad +	K*,S, other (1)
Bufo punctatus	red-spotted toad +	K*, other (2,3,6,7,9)
Bufo bufo	common toad +	K*, other (1,3,9)
Bufo marinus	cane toad +	K, other (3)
Andrias japonicus	Japanese giant salamander	K, S*, other (1,2,6)
Salamandra salamandra	fire salamander +	S*
Ambystoma tigrinum	tiger salamander	K,S, other (6)
Ambystoma mexicanum	axolotl (×3)	S*, other (1,2,3)
Class: Reptilia		
Bitis gabonica	Gaboon viper	K, S
Trimeresurus purpureomaculatus	mangrove pit viper	K, other (2)
Naja naja kaouthia	monocellate cobra	K, other (1,2)
Dipsosaurus dorsalis	desert iguana	K, other
Trachemys scripta elegans	red-eared terrapin	K, other (1,3)
Class: Aves		
Melopsittacus undulatus	budgerigar	K, S, other (1,8)
Psittacula sp.	parakeet	K, other (1)
Kakatoe sp.	cockatoo	K
Amazona sp.	Amazon parrot	K
Pulsatrix perspicillata	spectacled owl	K, other (1,6,9)
Spheniscus demersus	black-footed penguin	K, other (2,8)
Class: Mammalia		
Zaglossus bruijni	Bruijn's echidna	K*, other (1)
Macropus rufogriseus	red-necked wallaby (×3)	K*, other (1,2,9)
Orycteropus afer	aardvark	K*, S, other (2,6)
Zalophus californianus	Californian sea-lion	K*, other (3,9)

for 30 min, as in the first antibody stage. Sections were washed in PBS-Brij, covered with tetra-amino-biphenyl hydrochloride (diaminobenzidine), 0.1% in PBS, with 0.01% hydrogen peroxide, for 1–2 min, washed in water, counterstained with Mayer's haemalum, dehydrated and mounted in Piccolyte (Eastman Kodak, Liverpool, UK).

Controls

With each batch of sections stained by the immunohistological method, a section of normal human kidney was used as a positive control.

Human Tamm-Horsfall protein was prepared using the original method (Tamm and Horsfall 1952), with two cycles of precipitation by 0.58 M sodium chloride and redissolving in distilled water. From 3.4 litres of human urine, 24 mg of Tamm-Horsfall protein were prepared. 6 mg were coupled to 1 g cyanogen bromide-activated Sepharose using the manufacturers' instructions (Pharmacia, Uppsala, Sweden). The coupled Sepharose was put in a column connected to a pump and an ultraviolet recorder set at an absorbance of 280 nm. The sheep IgG anti-human Tamm-Horsfall protein that was used in the immunohistological method was diluted 1:2 in PBS and 2 ml were pumped through the column. Fractions of protein not retained on the column were collected and pooled. After washing the column with PBS, bound protein was eluted with a solution of 3 M potassium thiocyanate in double strength PBS and fractions collected. These were pooled and dialysed at 4° C against PBS with several changes over 24 h. Thus, 2 samples were prepared from the original antiserum: one, the material not retained by the human Tamm-Horsfall protein on the column, and the other, the material eluted after retention on the column.

Tamm-Horsfall protein from the leopard frog, *Rana pipiens*, was prepared by a modification of the original method (Tamm and Horsfall 1952). Twenty leopard frogs were kept in batches of 5, each batch in about 300 ml tap water, for 2 h. The water was filtered and then lyophilised. The dried material was suspended in 30 ml distilled water and centrifuged at 1700 *g* for 10 min. The supernatants were pooled. Sodium chloride was added to give a final concentration of 0.58 M NaCl and mixed well. The mixture was centrifuged at 2700 *g* for 30 min and most of the supernatant discarded, leaving only about 0.5 ml over a light, fluffy precipitate. Distilled water was added to 10 ml and mixed well. After centrifugation at 1700 *g* for 10 min, the supernatant was dialysed against distilled water at 4° C with several changes over 48 h, and then lyophilised. From 20 frogs, 100 µg of Tamm-Horsfall protein was prepared.

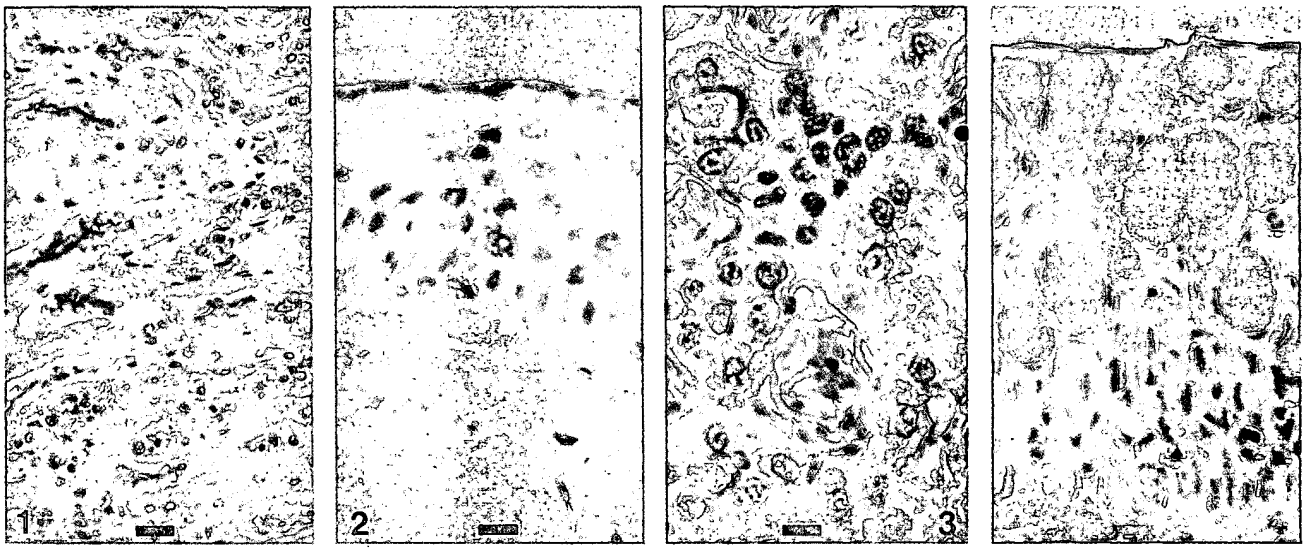

Fig. 1. Kidney of aardvark, *Orycteropus afer*, stained immunohistologically for Tamm-Horsfall protein, which is seen only in cells of the thick limb of the loop of Henle. *Bar*: 25 µm. ×200

Fig. 2. Skin of African clawed frog, *Xenopus laevis*, stained immunohistologically for Tamm-Horsfall protein, which is seen in the uppermost layer of the epidermis. *Bar*: 10 µm. ×450

Fig. 3. Kidney of American bullfrog, *Rana catesbeiana*, stained immunohistologically for Tamm-Horsfall protein, which is seen on the luminal border of distal tubules. *Bar*: 10 µm. ×450

Fig. 4. Skin of brown trout, *Salmo trutta*, stained immunohistologically for Tamm-Horsfall protein, which is seen in the uppermost layer of the epidermis. There is pigment just beneath the epidermis. *Bar*: 15 µm. ×350

Fig. 5. Gill of spotted dogfish, *Scyliorhinus canicula*, stained immunohistologically for Tamm-Horsfall protein, which is seen in the superficial layer. *Bar*: 7.5 µm. ×700

Anti-Tamm-Horsfall protein antibody was diluted to 1:600 in OA-PBS as in the immunohistological method. 0.6 ml of this diluted antibody was added to the 100 μg of dried frog Tamm-Horsfall protein, and the tube was rotated at room temperature for 30 min, while another 0.6 ml of the antibody was rotated similarly without Tamm-Horsfall protein. After centrifugation at 1700 *g* for 5 min, the samples of adsorbed and unadsorbed anti-Tamm-Horsfall protein antibody were used in the immunohistological method.

Results

The antiserum to Tamm-Horsfall protein reacted with the thick limb of the loop of Henle in the normal human kidney, and with the distal tubule in the kidney and superficial epidermis of the skin of the common frog, *Rana temporaria*, as had previously been shown (Howie et al. 1991).

Results are noted in Table 1. In animals tested of the class Mammalia, there was reactivity only with the thick limb of the loop of Henle in the kidney (Fig. 1). There was no reactivity with any of the tissues tested in animals of the classes Aves and Reptilia. In animals of the class Amphibia, most showed reactivity with the superficial layers of the epidermis (Fig. 2), and several showed reactivity with distal tubules in the kidney (Fig. 3), but with no other tissues. In animals of the classes Chondrichthyes and Osteichthyes, most had reactivity in the superficial layers of the skin (Fig. 4) and some in oral mucosa and gills (Fig. 5), but there was no reactivity in the kidney, nor in any other organ studied. Autolysis was often severe, especially in the organs of amphibians and fish that had been in water after death, and this is noted in Table 1.

Adsorption of the antiserum by the human Tamm-Horsfall protein coupled to Sepharose removed the reactivity with the human kidney and other mammalian kidneys, but did not completely abolish reactivity with fish or amphibian tissues. The antibody eluted from the column reacted with human and other mammalian kidneys and with fish and amphibian tissues. Adsorption of the antiserum with the frog Tamm-Horsfall protein removed the reactivity with amphibian tissues but not with human kidney. There was not enough frog Tamm-Horsfall protein to use the adsorbed antiserum on other tissues.

Discussion

We show that an antiserum to human Tamm-Horsfall protein, that reacts only with Tamm-Horsfall protein in human tissues (Howie 1987), stains on immunohistological study the kidney of other mammals and some amphibians, the skin of many amphibians and fish and the oral mucosa and gills of fish. There was no demonstrable reactivity with organs of reptiles or birds. Staining in the skin, oral mucosa and gills was confined to the most superficial layers, and in the kidney, to the thick limb of the loop of Henle in mammals, and the distal tubule in amphibians. A variable amount of autolysis probably explains the finding that not all fish showed reactivity in the skin and gills and not all amphibians showed reactivity in the skin and kidney. The use of dead animals was necessary to allow the widest possible range of species studied. The only other study on the phylogeny of Tamm-Horsfall protein failed to find immunohistological reactivity in the kidneys of marsupials, monotremes, birds, reptiles, amphibians or fish using rabbit antiserum to human Tamm-Horsfall protein (Wallace and Nairn 1971).

In the present study immunoreactivity was abolished by two substances. (1) Human and other mammalian reactivity was completely removed by a preparation of human Tamm-Horsfall protein coupled to Sepharose, although this did not completely remove reactivity with fish and amphibian tissues. (2) Amphibian reactivity was completely removed by a substance presumed to be leopard frog Tamm-Horsfall protein, although this did not completely remove reactivity with human kidney. This suggests that human and frog Tamm-Horsfall protein are similar but not identical. Some preparations of human Tamm-Horsfall protein appear to have more overlap with the leopard frog protein than others. For instance, the preparation that was used to immunise the sheep originally seems to have induced antibodies cross-reactive with the frog, that were completely removed by the previous sample of Tamm-Horsfall protein (Howie et al. 1991) but not by the sample used in the present study. Alternatively, it is possible that the chemical coupling of the human Tamm-Horsfall protein to Sepharose selectively obscured some of the more frog-like epitopes and allowed some anti-frog activity of the antiserum to escape binding on the immunoadsorption column. The antiserum used by Wallace and Nairn (1971), in contrast, appeared specific for the Tamm-Horsfall protein of placental mammals.

The function of Tamm-Horsfall protein is not known. As it is only found in the thick limb of the loop of Henle in the human kidney, which is impermeable to water and allows the kidney to pass dilute urine in the absence of antiduretic hormone, one possible function of the protein is to act as a waterproofing agent. This could be tested experimentally, perhaps by coating permeable membranes with the protein. The immunoreactive Tamm-Horsfall protein shown in fish and amphibian skin, fish oral mucosa and gills, and amphibian kidney does not necessarily have the same function as that in mammalian kidney, but these sites have a requirement for waterproofing. Freshwater fish, marine cartilaginous fish and amphibians are in a hypo-osmolar environment and are at risk of swelling from osmotic intake of water through the skin and gills, while marine bony fish are in a hyper-osmolar environment and are at risk of shrinkage through osmotic loss of water through the skin and gills. The kidney of amphibians cannot concentrate urine and produces hypo-osmolar urine. The diluting function of the kidney is in the distal tubule, which is therefore analogous to the human thick limb of the loop of Henle (Stoner 1977).

If this interpretation is correct, then Tamm-Horsfall protein appears strongly conserved in vertebrate evolution. It appears to have developed in the skin and gills of fish, persisted in the skin of amphibians and devel-

oped in the kidneys of amphibians, and persisted in the kidneys of mammals. Its apparent loss in the skin of mammals, reptiles and birds is perhaps because impermeability due to more extensive keratinisation became a better adaptation to limit water transfer across the skin. The kidney of reptiles and birds conserves water by passing urine that is supersaturated with uric acid, although some species are able to form dilute urine (Dantzler and Braun 1980). The kidneys of fish, especially of freshwater fish and elasmobranchs, which have a high urea concentration in the blood, pass dilute urine (Smith 1931). We consistently failed to show Tamm-Horsfall protein in the fish, reptile and bird kidneys and it appears therefore that in these animals either a mechanism different from that of amphibians and mammals is used to dilute urine or that the postulated Tamm-Horsfall protein-like waterproofing agent in the kidney is immunologically different from human Tamm-Horsfall protein.

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Bovine and Rodent Tamm-Horsfall Protein (THP) Genes: Cloning, Structural Analysis, and Promoter Identification

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We have isolated bovine and rodent cDNA and genomic clones encoding the kidney-specific Tamm-Horsfall protein (THP). In both species the gene contains 11 exons, the first of which is noncoding. Exon/intron junctions were analyzed and all were shown to follow the AG/GT rule. A kidney-specific DNase I hypersensitive site was mapped onto a rodent genomic fragment for which the sequence is highly conserved in three species (rat, cow, and human) over a stretch of 350 base pairs. Primer extension and RNase protection analysis identified a transcription start site at the 3′ end of this conserved region. A TATA box is located at 32 nucleotides upstream of the start site in the bovine gene and 34 nucleotides upstream in the rodent gene. An inverted CCAAT motif occurs at 65 and 66 nucleotides upstream of the start site in the bovine and rodent genes, respectively. Other highly conserved regions were noted in this 350 bp region and these are likely to be binding sites for transcription factors. A functional assay based on an in vitro transcription system confirmed that the conserved region is an RNA Pol II promoter. The in vitro system accurately initiated transcription from the in vivo start site and was highly sensitive to inhibition by α-amanitin at a concentration of 2.5 μg/ml. These studies set the stage for the further definition of *cis*-acting sequences and *trans*-factors regulating expression of the THP gene, a model for kidney-specific gene expression.

TAMM-HORSFALL protein (THP) is a glycoprotein with a molecular weight of 80 kDa, initially characterized by Tamm and Horsfall (1950) and subsequently purified from the urine of pregnant women by Muchmore and Decker (1985) as uromodulin. Pennica et al. (1987) showed that the two proteins were identical. THP is the most abundant protein in urine and is present in the kidneys of all placental mammals (Fletcher, 1972). Because one of our long-term research goals is to understand how gene expression is regulated in the kidney, we have chosen to use the THP gene as a model system for the following reasons. (1) The expression of THP is limited to the kidney in a nephron segment specific distribution. It is therefore a good example of a kidney-specific gene. Within the kidney, it is expressed only in the thick ascending limb of Henle's loop and in the distal convoluted tubule (Schenk et al., 1971). THP is produced in all mammalian species with a similar nephron-specific localization. The gene is therefore likely to have conserved *cis* regulatory sequences, and comparison of homologous regions from different species could implicate motifs that might bind tissue-specific transcription factors. (2) THP mRNA is present in high amounts (0.5–1% of message). The abundance of this message should greatly facilitate studies of gene regulatory sequences.

As a first step toward elucidation of the mechanisms that regulate kidney-specific gene expression, we have isolated and characterized the bovine and rodent THP genes. These animal homologues of the human THP gene are desirable due to the availability of kidney tissue that is required for chromatin structural studies and for making nuclear extracts

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(see below). In this article we describe the cloning of cDNAs containing the entire coding region and most of the noncoding region for bovine and rodent THP. The entire sequence of the cDNAs and the intron/exon structure of the two genes are also presented. We have identified a DNase I hypersensitive site in a highly conserved region that is also the promoter of this gene, as demonstrated by RNA mapping analysis and through an in vitro functional assay.

MATERIALS AND METHODS

Cloning, Restriction, and Sequence Analysis

cDNA libraries were in lambda gt10. The rodent library was a kind gift of Dr. G. Bell (University of Chicago). Standard procedures were used for cloning, restriction, and sequence analysis (Sambrook, 1989). Genomic libraries were in the lambda DASH II vector (Stratagene). cDNA and genomic inserts were cloned into pUC19 and pBluescript II SK, respectively.

RNA Isolation and Northern Analysis

RNA was isolated by the Chomczynski protocol (Chomczynski and Sacchi, 1987). Northern blotting and hybridization were as described (Current Protocols, 1991). Inserts from cDNA plasmids pBTHP1 and pRTHP1 were labeled by random priming (Feinberg and Vogelstein, 1983).

DNA Sequencing

The cDNA phage insert was subcloned in pUC19 and DNA sequencing was carried out using the chain-termination method with Sequenase (United States Biochemical).

DNase I Hypersensitive Site Analysis

Nuclei were isolated from 5 g of kidney and liver by mincing tissue in 25 ml of 0.25 M sucrose, 10 mM Tris HCl (pH 7.5), 5 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40. Tissue was homogenized in a Potter-Elvejem homogenizer and the nuclei were isolated by centrifugation. They were washed three times with the same buffer without NP-40 and resuspended at a DNA concentration of 1 mg/ml. DNase I was added to aliquots of the nuclei at 0, 1, 2, 5, and 10 μg/ml final concentration and incubation was carried out for 2 min at 25°C. One volume of stop solution (12.5 mM EDTA, 0.5% SDS, 500 μg/ml proteinase K) was added and incubation was carried out for 30 min at 37°C. Phenol-chloroform extractions were performed twice and a chloroform extraction was carried out before the DNA was precipitated with EtOH. The DNA was resuspended in TE and digested with Hind III, electrophoresed, and blotted onto nylon membranes. Prehybridization and hybridization was in 6 × SSC, 0.5% SDS, 100 μg/ml salmon sperm DNA, 5 × Denhardt's reagent (Current Protocols, 1991), and 50% formamide. A random printed, ^{32}P-labeled 300 bp intron 1 probe that was generated by PCR (diagrammed in Fig. 3) was used.

RNA Mapping Analysis

RNase protection assays were according to the method of M. Gilman (Gilman in Current Protocols, 1991). Total RNA (10 μg) was used for all reactions. For bovine THP, an EcoRI-Pst I 800 bp genomic fragment that contains the first exon was subcloned into pBluescript II SK+. The plasmid was digested with Ssp I and the 220 bp fragment was transcribed by T_3 RNA polymerase to give an antisense riboprobe. For rat THP, a PCR fragment from the rat conserved region −350 to +35 was cloned into the SmaI site of pBluescript II SK. The plasmid was cut with XhoI and transcribed with T_3 RNA polymerase to give an antisense riboprobe; 5 × 10^5 cpm were used for the hybridization.

Primer extension analysis was carried out as described by R. Kingston (Current Protocols, 1991) with specific exon 2 oligos BTHP4 and BTHP7 for bovine samples and RTHPRE36 and RTH5 for rat samples. mRNA was prepared by using oligo-dT columns (Boehringer Mannheim). Fewer nonspecific extensions resulted when 2 μg mRNA rather than 10 μg total RNA was used.

In Vivo Transcription

Extracts for in vitro transcription were made by the method of Gorski (1986) with the following modifications for kidney extracts: homogenization buffer 1 was supplemented with 1% nonfat milk, 5 μg/ml bestatin, 0.1 μg/ml leupeptin, 0.1 μg/ml pepstatin, 1 mM $NaMO_4$ (we found that inclusion of this phosphatase inhibitor was critical to the success of the procedure); homogenization buffer 2 contained the same supplement. Nuclear lysis buffer and nuclear dialysis buffer contained the same supplement but without the nonfat milk. Moreover, prior to homogenization, the kidneys were passed through a garlic press (Crate and Barrel worked best!).

The transcription reactions were essentially as

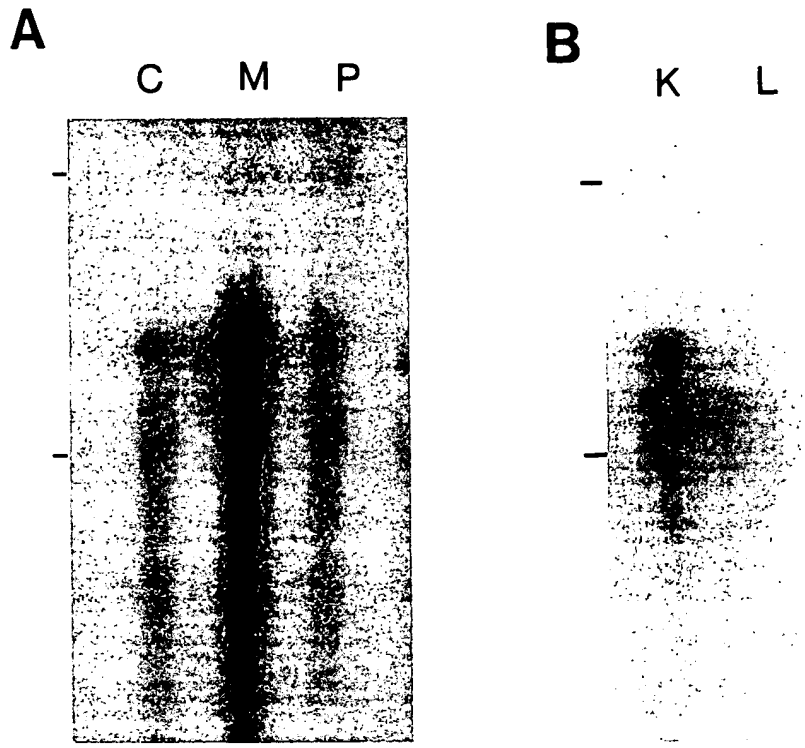


FIG. 1. (A) Northern blot of total cortical (C), medullary (M), and papillary (P) bovine RNA probed with the insert of pRTHP1. (B) Northern blot of total kidney (K) and liver (L) rat RNA probed with the same insert. Markers refer to 28S and 18S ribosomal sizes (~5 kb and ~2 kb, respectively).

described (Gorski et al., 1986). Template DNAs were prepared by a triple-spin $CsCl_2$ procedure and used at final concentrations of 50 μg/ml; 60 μg/ of protein was used per assay and α-amanitin was at 2.5 μg/ml.

RESULTS

Rodent and Bovine THP cDNA Homologues

A 550 bp BamH1-Hind III fragment from the human cDNA clone UM19 was used to screen rodent and bovine kidney cDNA libraries (Hession et al., 1987). Approximately 0.5% of plaques were positive in each library. The longest bovine clone, designated pBTHP1, had an insert of 2.5 kb whereas the longest rodent clone, pRTHP1, contained a 2.2 kb fragment. The inserts were used as probes for Northern analysis. As seen in Fig. 1A, the bovine probe hybridized strongly to a 2.6 kb transcript in outer medulla but weakly to cortex or papilla. The rodent probe hybridized to kidney but not liver (Fig. 1B).

pBTHP1 and pRTHP1 were fully sequenced (Fig. 2). pBTHP1 contains 1929 bp in its longest open reading frame and pRTHP1 contains 1932 bp leading to a predicted molecular weight of THP of ≈ 68 kDa (taking into account the 24 amino acidleader sequence cleaved from the precursor protein). Given the measured M.W. of ≈ 90 kDa, the carbohydrate moiety accounts for about 25% of the total. At the nucleotide level the bovine sequence shows 72.5% and 80.0% homology to the rodent and human sequences, respectively. Pennica et al. (1987) noted an unusually high number of CpG dinucleotides in the human third exon. This is also seen in the bovine third exon (63 CpGs). However, only 29 CpGs were noted in the rodent third exon, still a number greater than the predicted number due to CpG suppression, which is 18. The significance of this CpG cluster is not known.

Genomic Clones Spanning the Entire Rodent and Bovine THP Genes

Rat genomic clones were isolated by screening approximately 2×10^6 recombinant phage from a rodent genomic library (Stratagene) using the pRTHP1 cDNA insert as a probe. This screening yielded 16

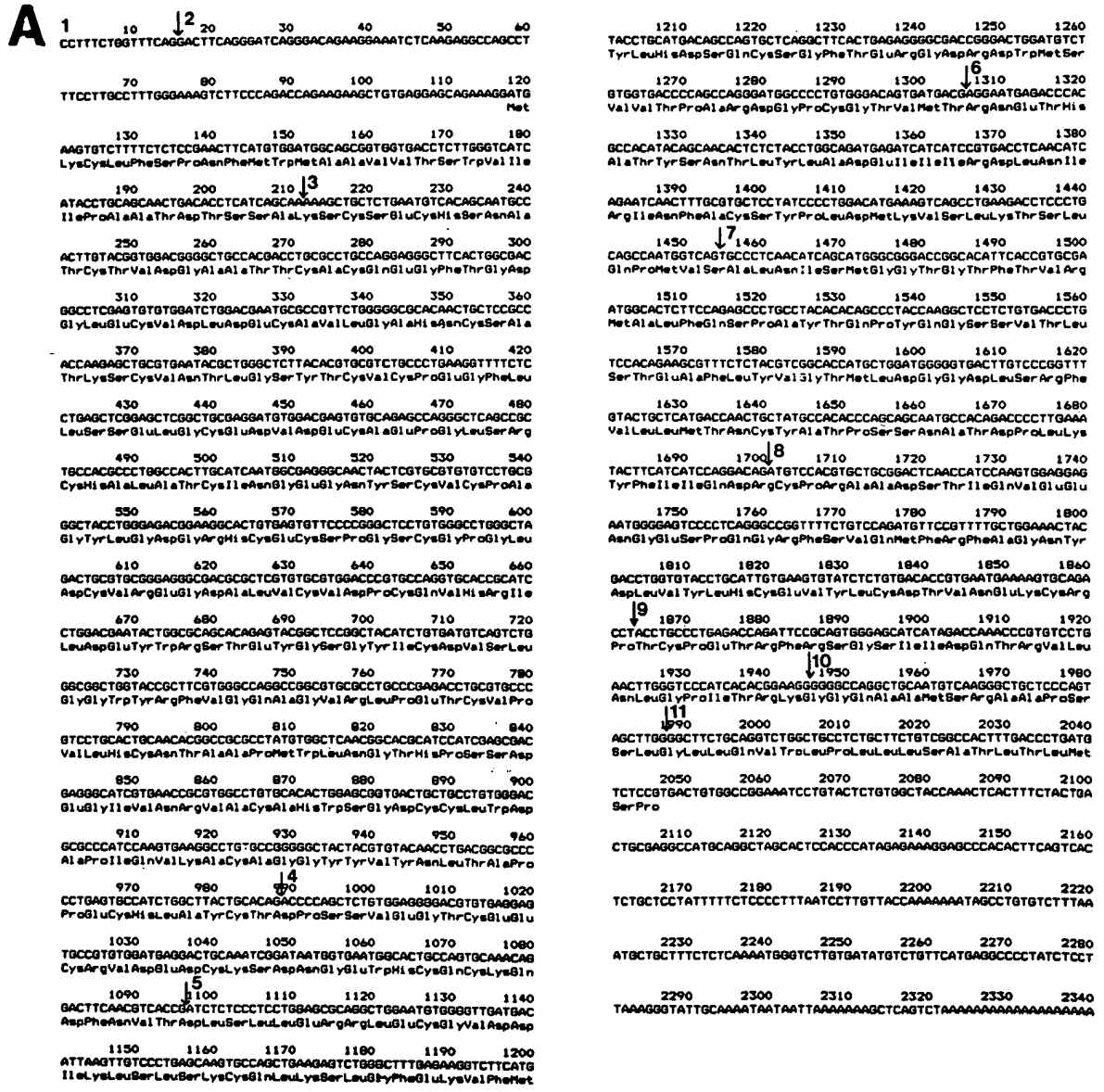


FIG. 2. (A) Complete sequence of the insert of pBTHP1. Exon boundaries have been denoted.

positive clones. The clones were further characterized and subgrouped by plaque hybridization with 17-mer oligos corresponding to selected exon sequences. Three clones were found to overlap and contained all 11 exons. Restriction blot analysis of these clones showed that the gene spans approximately 16 kb (Fig. 3B). The pBTHP cDNA insert was similarly used to screen 1×10^6 phage of a bovine genomic library (Stratagene). Out of eight positive clones, three were chosen for further structural analysis by restriction blot hybridization with four fragments of pBTHP1 (Fig. 3A).

The bovine gene spans 25 kb. Exon/intron junctions were assigned to both genes by sequence analysis using the junctions reported by Pennica et al. as a guide. All 10 exon/intron junction sequences follow the AG/GT rule (Breatnach and Chambon, 1981). The junctions show complete homology to the human sequences. Both the rat and bovine THP genes consist of 11 exons, the first of which is noncoding.

A Kidney-Specific DNase I Hypersensitive Site at the THP Gene Promoter

Because promoters are frequently associated with DNase I hypersensitive sites (Gross and Garrard,

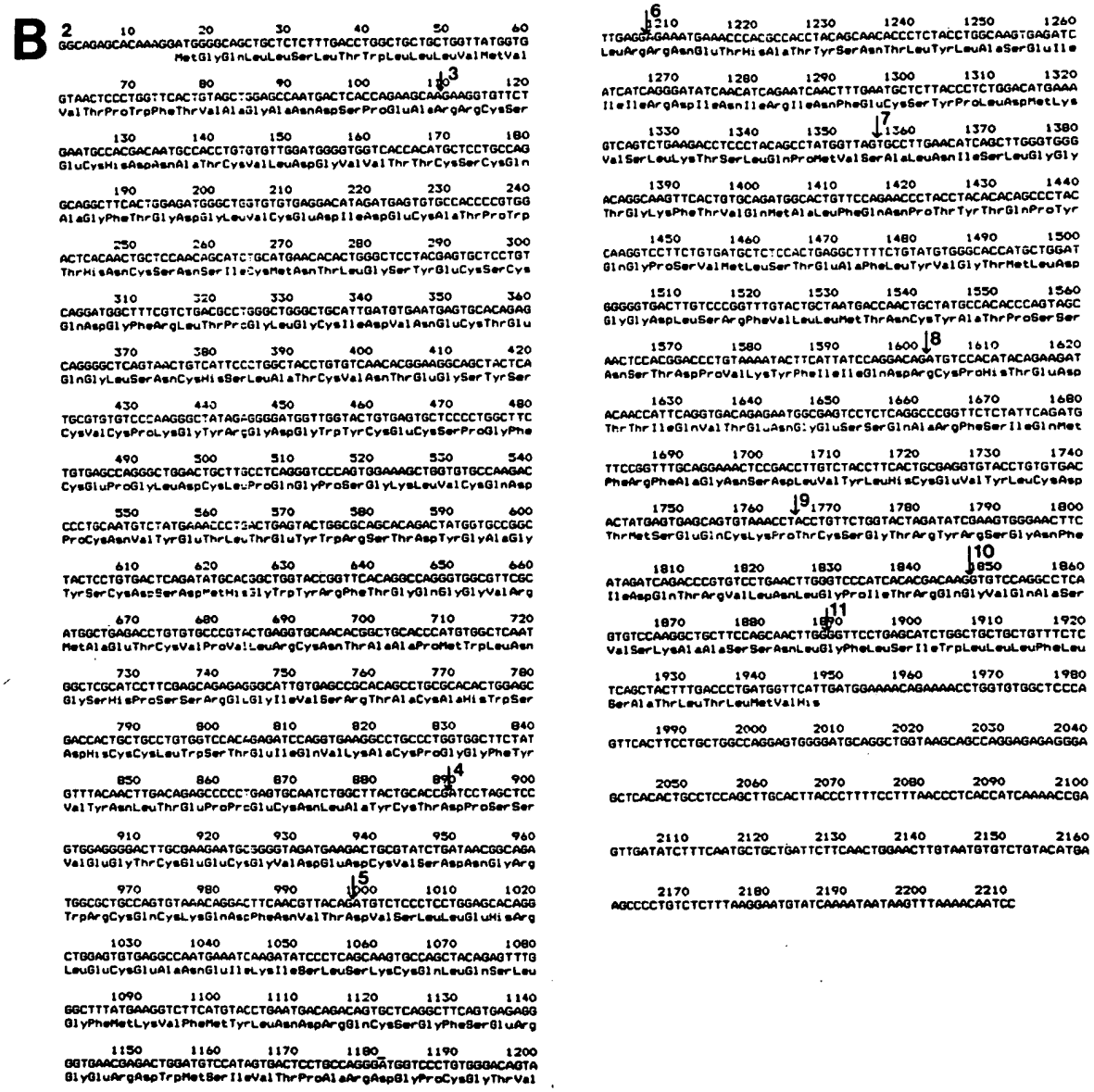


FIG. 2. (B) Complete sequence of the insert of pRTHP1.

1988), we searched for such sites in genomic fragments that were just upstream of the rodent cDNA 5′ end. Rat kidney nuclei were subjected to limited amounts of DNase I, then restricted for genomic Southern analysis. A genomic probe of 300 bp (derived by PCR) located in intron 2 was used (denoted in Fig. 3B as "P"). The expected 3 kb Hind III-Hind III fragment is reduced to 1.7 kb in a fraction of the nuclei (Fig. 4). This heterogeneity was anticipated because only about 10% of the cells in a whole kidney (those in the thick ascending limb and early distal nephron) are thought to express the THP gene. Importantly, the hypersensitive site is absent from rat liver nuclei subjected to the same manipulations and also from DNA isolated from a rat kidney directly (without an intermediate nuclear fractionation and DNase I digestion step). We sequenced the locus of the hypersensitive site and found striking homology between the rat sequence and sequence obtained from a region of a human genomic clone just upstream of the alternatively spliced exon "a" reported by Pennica et al. A rat probe spanning the hypersensitive site (derived through PCR) was then used to locate the homologous region in the bovine genomic clones. This sequence comparison is presented in Fig. 5. The conserved

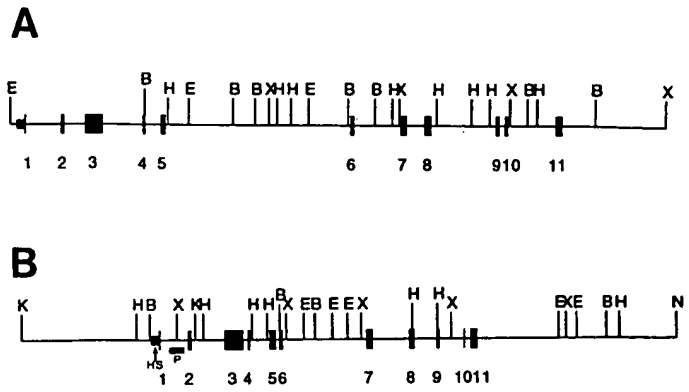


FIG. 3. Genomic organization of the bovine (A) and rat (B) THP gene. Boxes represent exons and have been numbered. The locus of the hypersensitive site in the rat clone has been marked as HS. Restriction sites are abbreviated: K, KpnI; E, EcoRI; H, HindIII; X, XbaI; B, BamHI; N, NcoI.

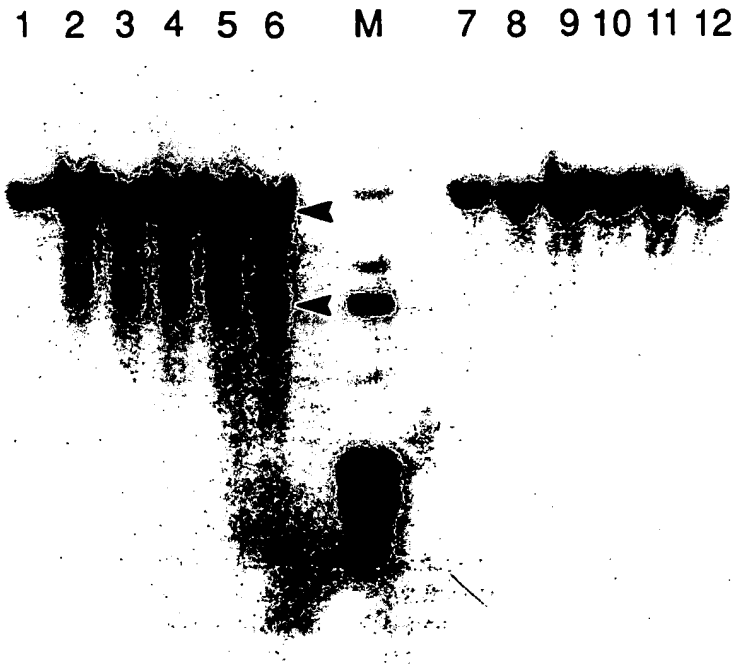


FIG. 4. DNase I hypersensitive site analysis. Lanes 1 and 7: DNA from rat kidney and liver (respectively) isolated without an intermediate nuclear purification step so as to minimize degradation. Lanes 2–6: DNA from kidney nuclei subjected to DNase I at concentrations of 0, 1, 2, 5, and 10 μg/ml. Lanes 8–12: DNA from liver nuclei subjected to 0, 1, 2, 5, and 10 μg/ml of DNase I. All DNAs were digested with HindIII. The probe is from intron 1, a nonrepetitive, PCR-generated 300 bp fragment (see the Materials and Methods section). The original 3 kb HindIII-HindIII band (top arrow) is reduced to 1.7 kb (lower arrow) in approximately 10% of the sample and only in samples derived from kidney nuclei. Lane M is a marker lane, with a "1 kb ladder" containing 1 kb marker DNAs and a Hinf I digest of pBR322 (Gibco, BRL).

region spanned approximately 350 bp in all three species and contained distinctive promoter hallmarks: a TATA box and an inverted CCAAT box (underlined).

RNA Mapping to Locate the Transcription Start Sites

Primer extension analysis was performed on bovine outer medullary RNA. Two oligo probes, BTHP4 and BTHP7, randomly chosen from exon 2 produced primer-extension products at 16 nucleotides upstream from nucleotide position 1 of the cDNA containing plasmid pBTHP1 (Fig. 6A). An RNase protection assay using a 220 bp fragment (Ssp I-Pst I), which contained the first exon, gave a protected nucleotide size at a range of 32–33 confirming the first exon size of 31 nucleotides as shown by the primer extension assay (Fig. 7A). When primer extension analysis was performed on rat kidney RNA using two different antisense primers RTHPRE36 and RTH5, separated by 57 bases, major extension products of 102 and 159 nt were detected (Fig. 6B). RNase protection analysis using a 385 bp antisense riboprobe spanning the conserved region (from nt 229 to 614, Fig. 5) gave a protected fragment of 30 bp (Fig. 7B). Liver RNA was negative by both methods. The results of the mapping studies in both species were in close agreement and identified a cap site 32 bp and 34 bp downstream of the TATA boxes underlined in Fig. 5.

In Vitro Transcription

To date, and despite extensive searching, we have not found a cell line expressing THP message. To begin to study cell type specific regulation of THP, we elected to attempt in vitro transcription from a kidney extract, initially derived from whole rat kidneys. Similar extracts from other organs (e.g., liver) have proven to be valuable reagents for studying determinants of tissue-specific transcription in vitro. Our kidney extracts retain the capacity to utilize the in vivo cap site and are sensitive to low concentrations of α-amanitin (2.5 μg/ml). We found that the production of transcriptionally competent nuclear extracts from kidney required protease inhibitors in addition to those described by Schliber (Gorski et al., 1988), and the phosphatase inhibitor sodium molybdate (see the Materials and Methods section).

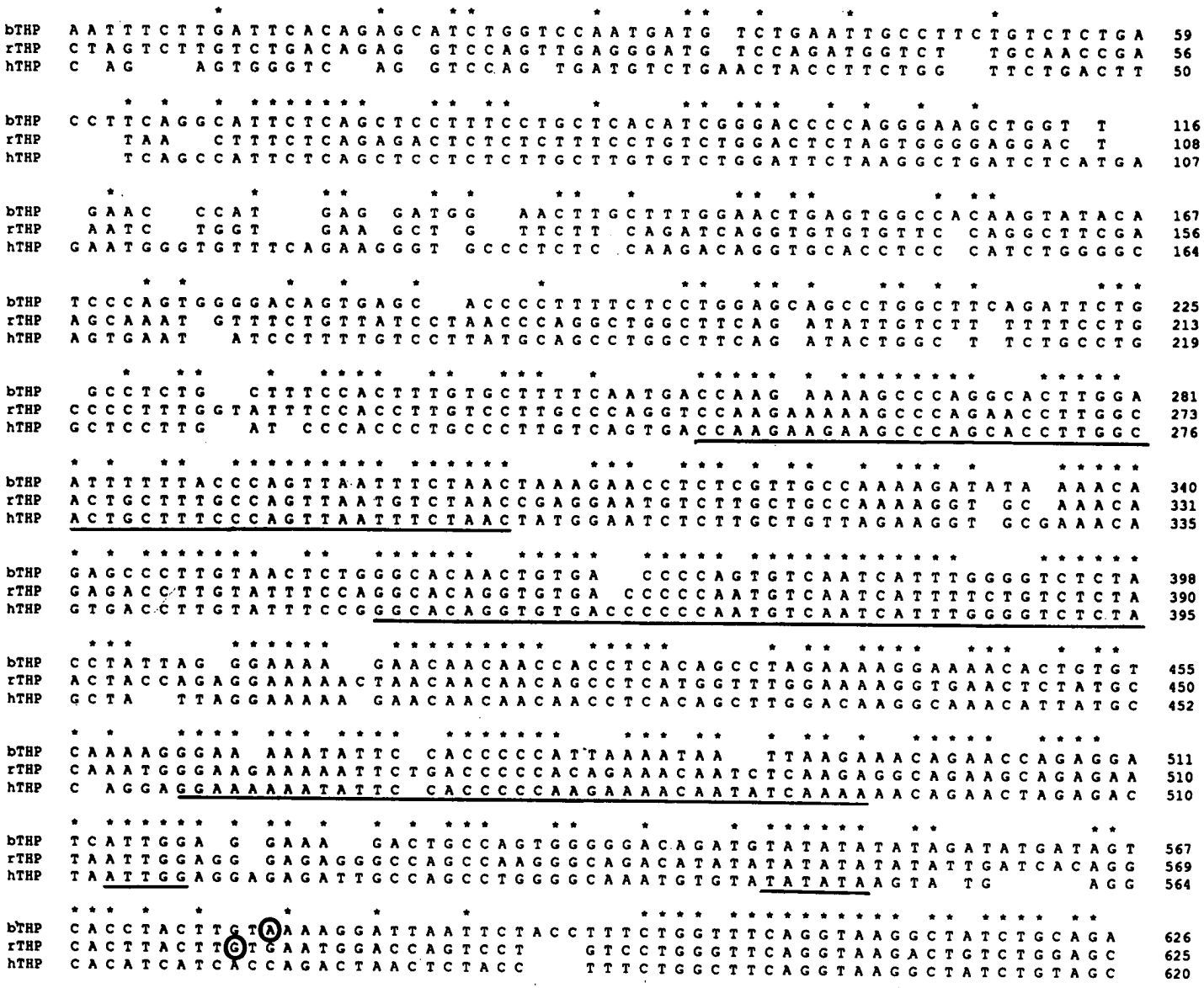
A rat THP promoter fragment from −1600 bp to −3 bp (relative to the assigned start site in the rodent gene, Fig. 5) was fused just upstream of the G-less cassette in pC_2AT (a kind gift of Dr. R. Roeder) to give pTHP380. Rat kidney nuclear extracts faithfully initiated transcription from the in vivo cap site (3 bp into the cassette) to produce the expected 380 bp transcript that is truncated by the chain-terminating guanosine nucleotide analog 3′-*O*-methyl GTP (lane 1, Fig. 8) In addition, the inclusion of α-amanitin at 2.5 μg/ml resulted in complete poisoning of transcription as is seen with other RNA Pol II promoters (lane 2, Fig. 8). A construct that has the orientation of the promoter fragment of pTHP380 reversed also gave no signal (data not shown). The results of this functional assay provide further proof that the 350 bp conserved region is the promoter for the THP gene.

DISCUSSION

One approach to the study of renal development is to define a hierarchical set of renal transcription factors that participate in a temporal and spatial cascade of transcriptional activation of kidney-specific genes, ultimately leading to the differentiated state. A tissue-specific marker gene can serve as a necessary springboard in such an approach. For example, much insight into the problem of hepatogenesis has been gained by studying the mechanisms that underlie liver-specific activation of the albumin gene (de Simone and Cortese, 1988).

As a first step towards elucidation of the mechanisms of transcriptional regulation of a kidney-specific gene, we have cloned and characterized the bovine and rat THP genes. Bovine and rat cDNA-clones were obtained by screening kidney cDNA libraries with a human THP cDNA fragment. Genomic clones were then isolated by using the cDNAs as probes. The location of exons on the genomic map along with a detailed analysis of the exon/intron junctions was a necessary first step in delineating the boundaries of the THP transcription unit. The promoter was identified through a combination of strategies that included DNase I hypersensitive site analysis, interspecies sequence comparison, and RNA mapping studies. That the promoter fragment identified by these means directs RNA Pol II-mediated transcription in vitro further authenticates its central role in the transcription unit of the THP gene.

A cell line that expresses the THP mRNA at the normally high in vivo levels does not exist. Therefore, we have chosen to work with the bovine and rat genes to characterize gene regulatory elements by species comparisons, using transgenic mice and by biochemical approaches. The latter strategy requires large quantities of kidney nuclei that are readily available from animals. The cow kidney offers the additional advantage of further dissection of the


bTHP AATTTCTTGATTCACAGAGCATCTGGTCCAATGATG TCTGAATTGCCTTCTGTCTCTGA 59
rTHP CTAGTCTTGTCTGACAGAG GTCCAGTTGAGGGATG TCCAGATGGTCT TGCAACCGA 56
hTHP C AG AGTGGGTC AG GTCCAG TGATGTCTGAACTACCTTCTGG TTCTGACTT 50
bTHP CCTTCAGGCATTCTCAGCTCCTTTCCTGCTCACATCGGGACCCCAGGGAAGCTGGT T 116
rTHP TAA CTTTCTCAGAGACTCTCTCTTTCCTGTCTGGACTCTAGTGGGGAGGAC T 108
hTHP TCAGCCATTCTCAGCTCCTCTCTTGCTTGTGTCTGGATTCTAAGGCTGATCTCATGA 107
bTHP GAAC CCAT GAG GATGG AACTTGCTTTGGAACTGAGTGGCCACAAGTATACA 167
rTHP AATC TGGT GAA GCT G TTCTT CAGATCAGGTGTGTGTTC CAGGCTTCGA 156
hTHP GAATGGGTGTTTCAGAAGGGT GCCCTCTC CAAGACAGGTGCACCTCC CATCTGGGGC 164
bTHP TCCCAGTGGGGACAGTGAGC ACCCCTTTTCTCCTGGAGCAGCCTGGCTTCAGATTCTG 225
rTHP AGCAAAT GTTTCTGTTATCCTAACCCAGGCTGGCTTCAG ATATTGTCTT TTTTCCTG 213
hTHP AGTGAAT ATCCTTTTGTCCTTATGCAGCCTGGCTTCAG ATACTGGC T TCTGCCTG 219
bTHP GCCTCTG CTTTCCACTTTGTGCTTTTCAATGACCAAG AAAAGCCCAGGCACTTGGA 281
rTHP CCCCTTTGGTATTTCCACCTTGTCCTTGCCCAGGTCCAAGAAAAAGCCCAGAACCTTGGC 273
hTHP GCTCCTTG AT CCCACCCTGCCCTTGTCAGTGACCAAGAAGAAGCCCAGCACCTTGGC 276
bTHP ATTTTTTACCCAGTTAATTTCTAACTAAAGAACCTCTCGTTGCCAAAAGATATA AAACA 340
rTHP ACTGCTTTGCCAGTTAATGTCTAACCGAGGAATGTCTTGCTGCCAAAAGGT GC AAACA 331
hTHP ACTGCTTTCCCAGTTAATTTCTAACTATGGAATCTCTTGCTGTTAGAAGGT GCGAAACA 335
bTHP GAGCCCTTGTAACTCTGGGCACAACTGTGA CCCCAGTGTCAATCATTTGGGGTCTCTA 398
rTHP GAGACCTTGTATTTCCAGGCACAGGTGTGA CCCCCAATGTCAATCATTTTCTGTCTCTA 390
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bTHP CCTATTAG GGAAAA GAACAACAACCACCTCACAGCCTAGAAAAGGAAAACACTGTGT 455
rTHP ACTACCAGAGGAAAAAACTAACAACAACAGCCTCATGGTTTGGAAAAGGTGAACTCTATGC 450
hTHP GCTA TTAGGAAAAA GAACAACAACAACCTCACAGCTTGGACAAGGCAAACATTATGC 452
bTHP CAAAAGGGAA AAATATTC CACCCCCATTAAAATAA TTAAGAAACAGAACCAGAGGA 511
rTHP CAAATGGGAAGAAAAATTCTGACCCCCACAGAAACAATCTCAAGAGGCAGAAGCAGAGAA 510
hTHP C AGGAGGAAAAAATATTC CACCCCCAAGAAAACAATATCAAAAAACAGAACTAGAGAC 510
bTHP TCATTGGA G GAAA GACTGCCAGTGGGGGACAGATGTATATATATAGATATGATAGT 567
rTHP TAATTGGAGG GAGAGGGCCAGCCAAGGGCAGACATATATATATATATTGATCACAGG 569
hTHP TAATTGGAGGAGAGATTGCCAGCCTGGGGCAAATGTGTATATATAAGTA TG AGG 564
bTHP CACCTACTTGTAAAAGGATTAATTCTACCTTTCTGGTTTCAGGTAAGGCTATCTGCAGA 626
rTHP CACTTACTTGTGAATGGACCAGTCCT GTCCTGGGTTCAGGTAAGACTGTCTGGAGC 625
hTHP CACATCATCACCAGACTAACTCTACC TTTCTGGCTTCAGGTAAGGCTATCTGTAGC 620

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FIG. 5. Sequence alignment of promoters from three species: rat, cow, and human. The start sites are circled. TATA and inverted CCAAT boxes have been underlined, as have conserved regions at nucleotides 258–300, 359–400, and 468–487.

outer medulla to enrich for tissue-containing THP message at high amounts, and we have recently also successfully produced in vitro transcription competent extracts from this source (data not shown). The kidney nuclei served a twofold purpose. First, the nuclei are a source of the THP gene in its natural chromatin context; this allowed us to identify a kidney-specific DNase I hypersensitive site and to thereby locate the promoter. Second, the nuclei are a source of nuclear protein that could be used to carry out DNA binding studies (data not shown) and in vitro transcription.

The identification of the cap site proved complicated. The rat cDNA clone rTHP-1 ends to 16 bp upstream of the initiator methionine codon. Primer extension with two gene-specific primers indicated that the rat cap site was 68 bp upstream of the initiator codon. The rat extension product was not likely to be contiguous with the genomic sequence due to two reasons: (1) RNase protection analysis using an antisense riboprobe in this region gave a protected fragment that had its 5′ end only 39 bp upstream of the initiator codon (data not shown); this region has a consensus splice acceptor sequence; (2) the sequences upstream of the putative cap site—were the primer extension product to be contiguous with the genomic sequence—might have contained easily identifiable promoter elements. Because this was not the case, it seemed likely that a small exon having a maximum length of 30 bp existed somewhere upstream in the gene.

Pennica et al. (1987) isolated two classes of human THP cDNAs. The first class has a 25 bp exon that they refer to as exon "a" at the 5′ end. The second class has as its 5′ end the region just upstream of and contiguous with the second exon of the first class of cDNA. They refer to this as exon "b" and propose an alternative splicing event that gives rise to the two classes. We performed primer extension analysis using a human exon 2-specific primer on human RNA and found two extension products that agree with the boundaries of the cDNA ends of Pennica et al. (data not shown). However, both the rat and bovine THP mRNAs are associated with only one 5′ end. We think that this species-specific variation arises because a fraction of the human THP RNAs retain a region as the 5′ end (due to an abnormal splicing event) that in the other two species is an intron. We favor this explanation over the possibility that the human gene is associated with an additional promoter located upstream of exon "b" because there are no well-conserved promoter elements there and because that region does not serve as a promoter in vitro (data not shown).

For these reasons, it seemed likely that the expected 30 bp first exon in the rat gene would be the homologue of the human exon "a." If this were indeed the case, we expected extensive interspecies homology in the putative promoter regions upstream of these first exons. However, it proved difficult to locate the homologue of exon "a" on the rat gene by hybridization, due to the small size of the oligomer and its strong hairpin structure. Instead, the clue to the location of the rat homologue of exon "a" came from the mapping of a kidney-specific DNase I hypersensitive site, located approximately 700 bp upstream of the splice acceptor site of the rat exon 2. Importantly, DNA sequencing of a 350 bp region covering the hypersensitive site revealed an 80% homology with the genomic sequence upstream of human exon "a." The bovine gene contains the same conserved 350 bp region, and the homology between the bovine and the rat region is 72% (see Fig. 5).

RNase protection analysis identified a 5′ end in the rat gene that agreed precisely with the primer extension result and that accounted for the 30 additional bases (see Fig. 7). The bovine primer extension and RNase protection results further agreed with the rat RNA mapping.

The promoter region is associated with a TATA element at −34 and an inverted CCAAT element at −65. In addition, three highly conserved elements were seen at rodent nucleotides 258–300, 359–400, and 468–487 (underlined in Fig. 5).

We wished to see whether the THP promoter would function in an in vitro transcription system because the absence of a cell line prevented us from transient assay testing. A G-less cassette reporter (pTHP 380) gave an accurate size transcript of 380 bp. Furthermore, the inclusion of α-amanitin at 2.5 μg/ml completely abolished transcription, as would be expected of a Pol II promoter. These results show that the conserved region can serve faithfully as a Pol II promoter in an in vitro system. Future experiments will examine the role of the individual conserved elements through the use of this in vitro system. Ultimately, reporter constructs in transgenic mice will reveal the precise elements required for high-level, kidney-specific expression of the THP


A
A C G T 1 2
GGAAAGACCAAA

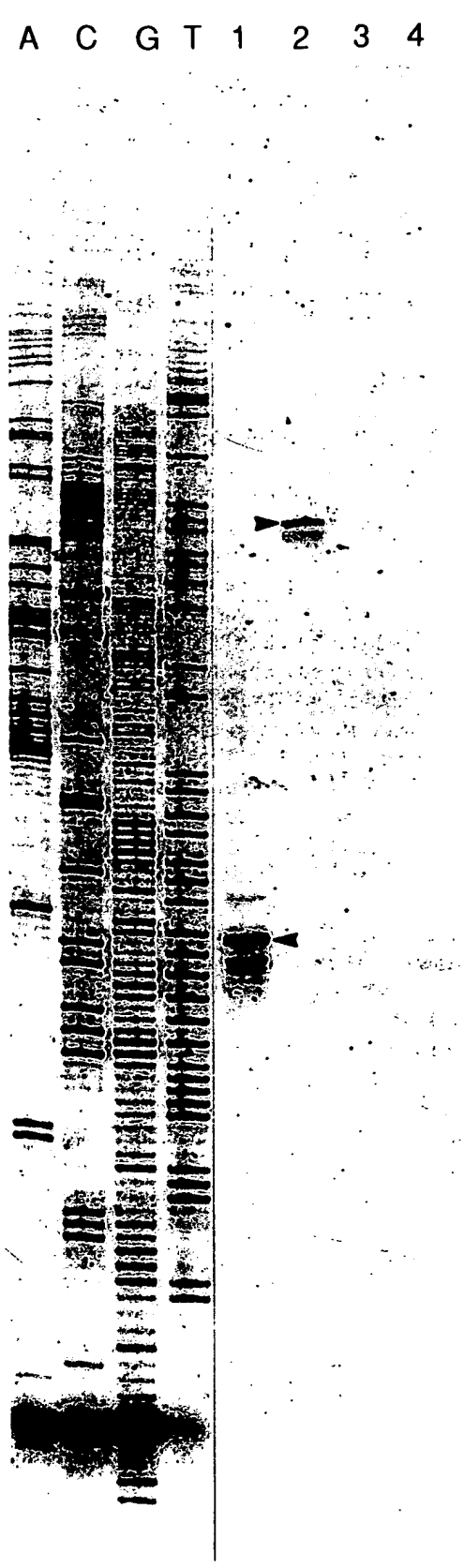
B
A C G T 1 2 3 4

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FIG. 6. (A) Primer extension of bovine kidney outer medullar RNA (20 μg) (lane 1) and tRNA (lane 2) with primers, bTHP4 and bTHP7 (not shown), randomly chosen from exon 2. The major extension product identified is 16 nucleotides upstream from the 5′ end of bTHP1. A sequencing ladder was produced with bTHP1 and the same oligo primers. (B) Primer extension analysis of rat kidney mRNA (2 μg) with primers RTHPRE36 (lane 1) and RTH5 (lane 2). Lanes 3 and 4 are extensions using rat liver mRNA (2 μg) and primers RTHPRE36 and RTH5, respectively. The major extension products (102 nt in lane 1 and 159 nt in lane 2) are marked by arrows. A sequencing ladder was run next to the reactions for sizing purposes.

message. With the cloning of THP genomic fragments containing all the exons as well as the promoter and considerable 5′ flanking sequence, we anticipate performing these experiments in the future. Finally, because virtually nothing is known about kidney-specific gene expression and nephron segment-specific expression, the studies reported here initiate a data base on this topic with implications to understanding kidney growth and differentiation.

ACKNOWLEDGEMENT

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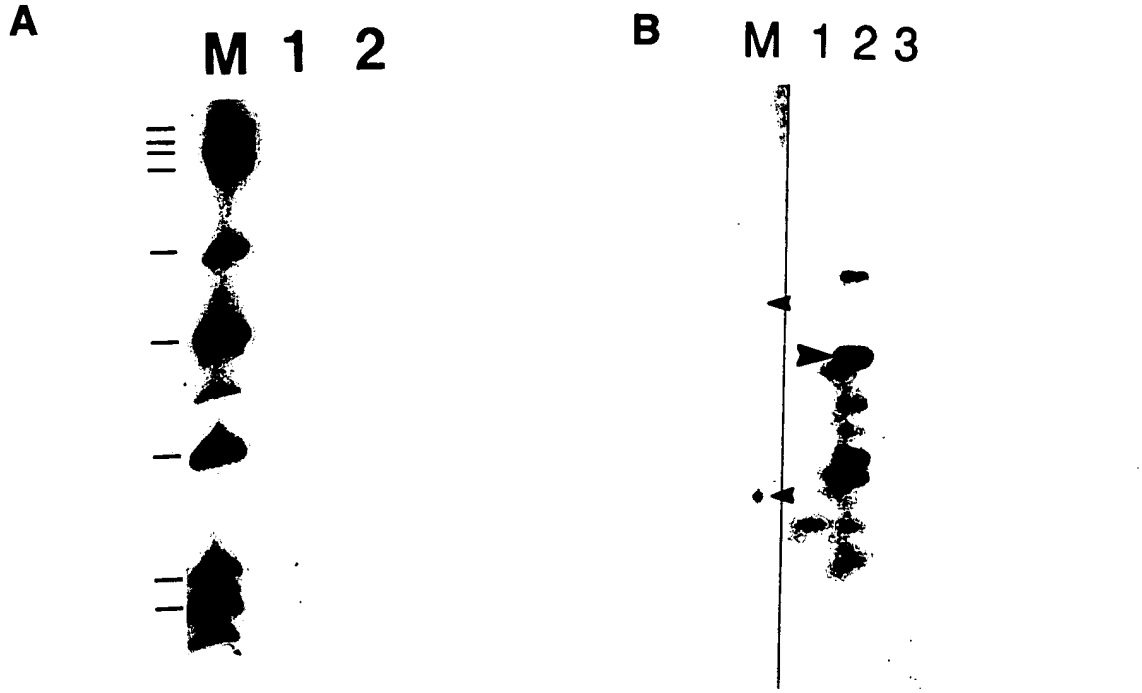

FIG. 7. (A) RNase protection assay for the bovine THP. Lane 1: bovine outer medulla RNA. Lane 2: tRNA. EcoRI-Pst I 800 bp genomic fragment that contains the first exon was subcloned into pBluescript II. The plasmid was digested with Ssp I, and the 220 bp antisense fragment was transcribed and used for hybridization. The arrow indicates a DNA nucleotide size range of 32–33, consistent with the first exon size of 31 nt, as shown by the primer extension assay. Bars indicate nucleotide sizes of 711, 489, 404, 364, 242, 190, 147, 118, 110, 67, 57, 34, 26, from the top. (B) Rnase protection analysis of rat samples using an antisense riboprobe from −350 to +35. Lane 1: liver total RNA (10 μg). Lane 2: kidney total RNA (10 μg). Lane 3: 10 μg tRNA. The top arrow in the marker lane points to 34 nt; the bottom arrow to 26 nt. The major transcript end (arrow in lane 2) is at 30 nt.

FIG. 8. In vitro transcription using kidney nuclear extracts. A total of 1 μg of template and 60 μg of nuclear protein were used for both reactions. Lane 1: pTHP380. Lane 2: pTHP380 with 2.5 μg/ml α-amanitin.

GENE EXPRESSION

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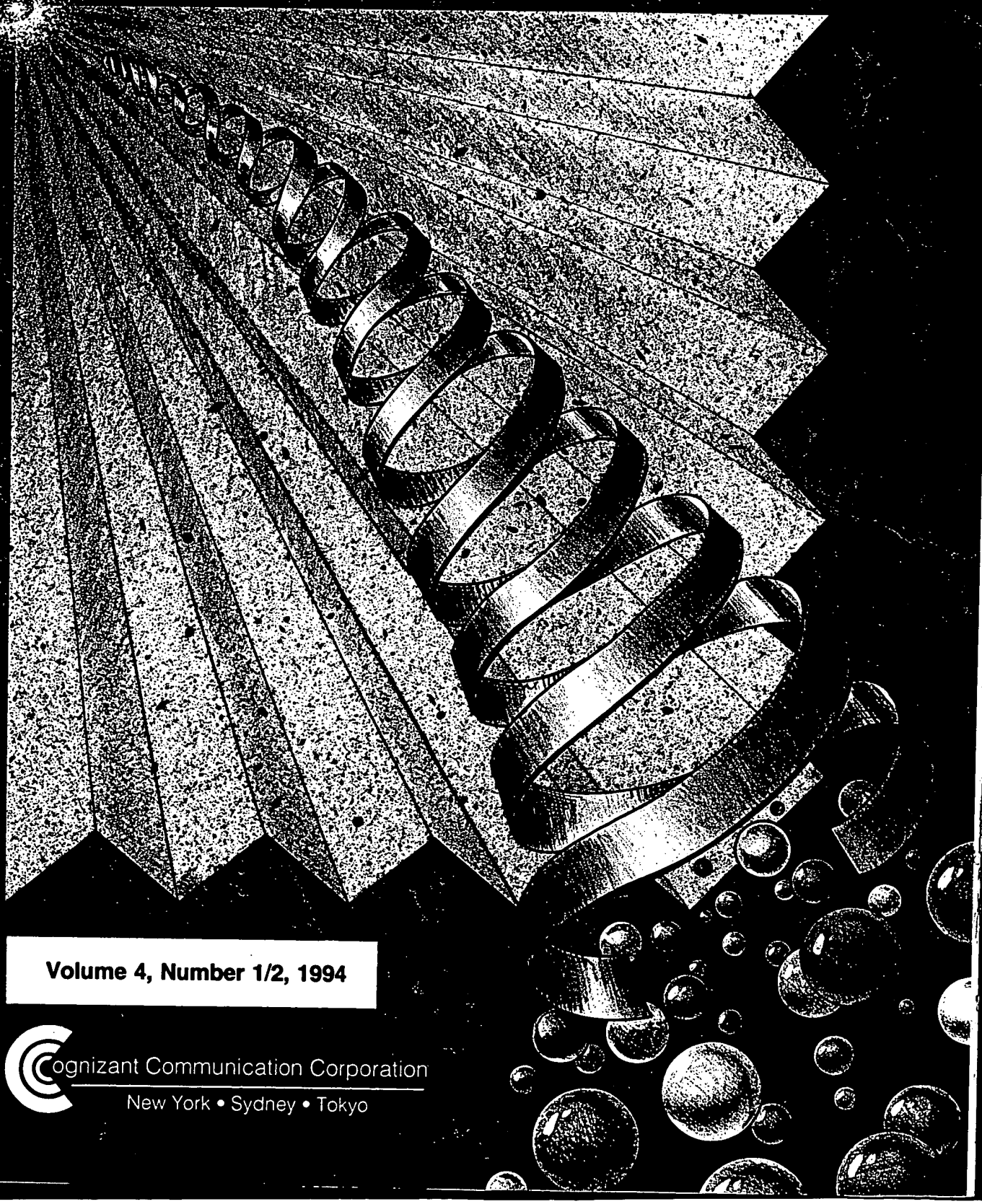

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BOVINE ALPHA$_{s1}$-CASEIN GENE SEQUENCES DIRECT HIGH LEVEL EXPRESSION OF ACTIVE HUMAN UROKINASE IN MOUSE MILK

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We have produced a line of transgenic mice carrying a hybrid bovine α_{s1} casein/human urokinase gene. Bovine α_{s1}-casein gene regulatory sequences specifically direct expression of the human urokinase gene in lactating mammary tissue from these mice. Urokinase is a 54 kD protein with 9 disulfide bonds that is normally synthesized in the kidney; however, the casein/urokinase transgenic mice secrete active human urokinase into their milk at concentrations of 1-2 mg/ml. The mice show no other abnormalities. They give birth to, and nurse, normal sized healthy litters. Thus it is possible to produce high concentrations of a large, cysteine rich, non-milk protein in the milk of transgenic animals. This line of transgenic mice provides a model for the eventual production of transgenic farm animals producing high levels of recombinant proteins in their milk.

Transgenic farm animals that specifically secrete the products of cloned genes into their milk may eventually provide an alternative to the *in vitro* cell culture methods now used for the production of recombinant proteins. We have generated a mouse model for such an *in vivo* expression system. These mice produce milk containing high levels of human urokinase, a plasminogen activator that is normally synthesized in the kidney and secreted into the urine. Several other groups have reported mammary specific gene expression in transgenic mice. Simmons et al.[1] generated transgenic mice expressing an intact sheep β-lactoglobulin gene in mouse milk at levels up to five-fold higher than found in sheep milk. Regulatory sequences from another milk specific gene, the mouse whey acidic protein, have been used to direct the expression of two nonmilk proteins in the mammary glands of lactating transgenic mice: a human HA-*ras* oncogene product[2] and human tissue plasminogen activator[3,4]. One of these proteins, human tissue plasminogen activator, was detected in the milk of transgenic animals. Six independently derived lines produced milk containing less than 0.5 μg/ml of the heterologous protein, while one line produced milk containing 50 μg/ml. We have used regulatory sequences from a different milk specific gene, the bovine α_{s1}-casein gene, to direct the expression of human urokinase, a non-milk protein, at a level of 1-2 mg/ml in the milk of transgenic mice.

RESULTS

Construction of a milk specific expression vector. The α_{s1}-casein gene is one of the most actively transcribed genes in bovine mammary tissue[5]. α_{s1}-Casein is the major protein component of cow milk, at 13 mg/ml it accounts for over 30% of the total protein[6]. We isolated genomic clones encompassing the α_{s1}-casein gene by probing a bovine cosmid library with synthetic oligonucleotides based on published cDNA sequences[5].

We used genomic sequences from two of these cosmid clones to construct a mammary specific expression vector, pCAS1288 (Fig. 1), that contains bovine α_{s1}-casein upstream and downstream sequences joined by a synthetic linker containing 2 rare restriction sites, XhoI and NotI.

FIGURE 1 Structure of the mammary specific expression vector pCAS1288 and the hybrid casein/urokinase gene pCAS1295. Abbreviations: poly A, polyadenylation site of the α_{s1}-casein gene; Xho/Sal, XhoI and SalI sites destroyed by fusion; B, BamHI; E, EcoRI; G, BglII; H, HindIII: K, KpnI; M, SmaI; X, XhoI.

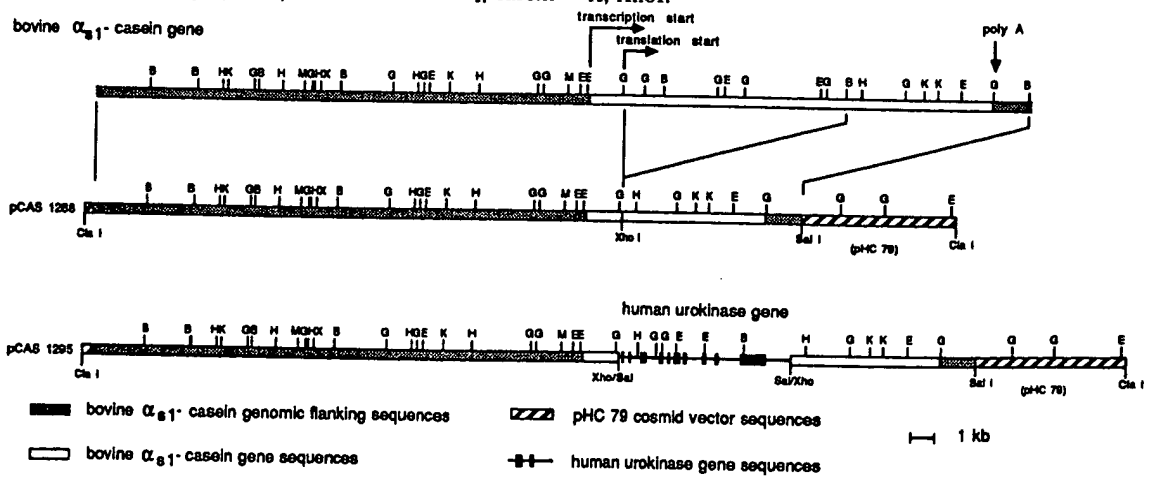

FIGURE 2 Northern blot of total RNA isolated from transgenic mouse tissue. The washed blot was exposed to x-ray film for 4 hours; a 2 day exposure revealed no hybridization signal in any lane except the mammary lane, data not shown.

The 5′ fragment consists of 21 kb of upstream sequences together with the first exon and that portion of the second exon preceding the translation start site; the 3′ fragment consists of the exons encoding the COOH-terminal half of the protein and the polyadenylation signal, together with 2 kb of downstream flanking sequences. To use this expression vector, a coding sequence can be cloned into the XhoI or NotI site. The coding sequence is thus provided with *cis*-acting bovine casein promoter and polyadenylation sequences. ClaI and SalI sites on either side of the casein genomic fragments can then be used to isolate the resulting chimeric construct from most of the bacterial vector sequences prior to microinjection into embryos (275 bp of the plasmid tetracycline resistance gene remains attached to the upstream bovine flanking sequences).

Construction of a reporter gene construct. We used the gene encoding human urokinase to determine if the bovine regulatory sequences in pCAS1288 could drive the expression of a heterologous gene in mammary tissue. We chose urokinase as a test gene for two reasons: (i), urokinase activity is easily measured, allowing us to determine if the enzyme is produced in an active form; and (ii), a large cysteine rich protein such as urokinase (naturally occurring urokinase is secreted as a 54 kD protein containing 9 disulfide bonds) rigorously tests the ability of the mammary gland expression system to provide a suitable environment for the correct folding of heterologous proteins.

We cloned the human urokinase gene from a cosmid library using synthetic oligonucleotides based on published sequences. We then subcloned a 7.5 kb ApaI fragment containing the entire coding portion of the urokinase gene, including 31 nucleotides upstream of the transcription start site together with 1 kb downstream from the polyadenylation site, converting the ApaI ends to SalI ends with synthetic oligonucleotide adapters. The hybrid casein/urokinase plasmid, pCAS1295 (Fig. 1), was constructed by cloning the resulting 7.5 kb SalI fragment into the central XhoI site of pCAS1288.

Generation of transgenic mic . We injected purified pCAS1295 insert into the pronuclei of fertilized (C57BL/6J × CBA/J)F_2 mice and transferred the surviving embryos into pseudopregnant females as described by Hogan et al.[7]. Eighteen embryos survived to term. Southern blot analysis of DNA isolated from tail tissue from each of these mice identified three transgenic founder animals (male #1203, female #1205, and male #1468). We estimate from the intensity of the Southern blot hybridization signal (data not shown) that mouse #1203 contained approximately 2 copies of the inserted transgene per cell, while mice #1205 and #1468 contained approximately 10 and 100 copies per cell respectively. The three founder mice were bred with nontransgenic (C57BL/6J × CBA/J)F_1 mice to produce second generation transgenics. Mouse #1468, which carried approximately 100 copies of the transgene per cell, produced 38 offspring; none of these offspring were transgenic. Mouse #1205, containing 10 copies per cell, produced 23 offspring; again all of the offspring were negative. Mouse #1203, which had only two copies integrated per cell, was the only founder animal to pass the transgene on to its progeny; it produced 23 offspring, 15 of which were positive. We intercrossed the positive offspring of mouse #1203 and identified mice that were hemizygous and homozygous for the transgene by southern blotting.

RNA analysis. Total RNA from the brain, kidney, liver, lung, mammary gland, pancreas, spleen, and thymus of a lactating mouse homozygous for the transgene was separated by gel electrophoresis, blotted it onto a nylon membrane, and bound RNA hybridized with a ^{32}P-labelled cDNA clone of human urokinase (Fig. 2). Mammary gland is the only tissue in which expression of RNA species that hybridize to the human urokinase cDNA probe was detected. The human urokinase cDNA probe does not cross-hybridize with any endogenous transcripts in mammary tissue RNA isolated from non-transgenic control mice (data not shown). Lactating mammary tissue from transgenic mice contains three transcripts that cross hybridize with human urokinase: a predominant 5 kb species and two minor species of 3.8 kb and 2.7 kb. In contrast, the transcript encoding urokinase in human kidney carcinoma cells is approximately 2.5 kb in length[8]. The larger mRNA species in the transgenic mammary tissue may represent incompletely spliced forms of the urokinase transcript, or fusions of human urokinase exons with the bovine casein genomic sequences located downstream in the injected chimeric construct. The latter possibility could result from inefficient recognition of the urokinase polyadenylation signal in this context. We do not yet know if all three of the observed transcripts are translated to produce functional urokinase. If a majority of the active urokinase secreted into the milk is synthesized from one of the less abundant mRNA species, then it may be possible to design more efficient expression vectors by removing the downstream casein exons.

Protein analysis. We analyzed milk from 3 control and 7 transgenic animals to determine if active human urokinase is being secreted into the milk of mice carrying the pCAS1295 insert. The level of enzymatically active urokinase was estimated using a casein/plasminogen gel assay[9]. Briefly, serial dilutions of the sample are tested for their ability to induce casein proteolysis in an agarose gel impregnated with casein and plasminogen. Plasminogen activator in the sample diffuses into the gel, converting inactive plasminogen to active plasmin, which then cleaves the casein. Zones of casein proteolysis appear as clear discs in the otherwise turbid gel. Comparison of plasminogen activator activity in the milk of transgenic and control non-transgenic animals to the activity of commercially purified human urokinase consistently measured plasminogen activator activity in the milk of transgenic animals at a level comparable to 1-2 mg/ml human urokinase

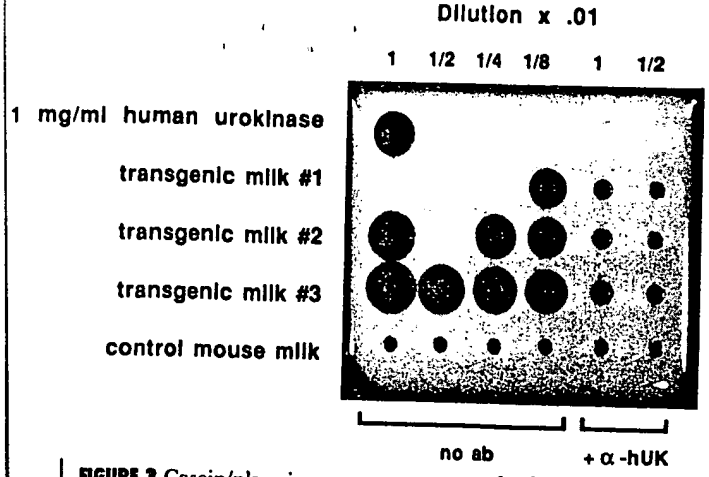

FIGURE 3 Casein/plasminogen agarose assay for human urokinase activity. Two fold serial dilutions of diluted milk from control and transgenic mice compared to human urokinase standards. Mouse milk was diluted 100 fold with phosphate buffered saline (PBS). Commercially prepared human urokinase (high molecular weight urokinase, catalog #125, American Diagnostica Inc. New York, NY) was diluted to 10 μg/ml in PBS. Each sample was then serially diluted 3 times with equal volumes of PBS. Ten μl of each dilution was added to wells cut into a casein/plasminogen agarose gel as described by Nagamine et al.[9]. The first 2 dilutions of each sample were plated a second time in the presence of 10 ng of a monoclonal antibody that specifically blocks the activity of human urokinase (American Diagnostica Inc., New York, NY, catalog #394). Zones of plasminogen activator activity appear as cleared regions of casein hydrolysis. Transgenic milk sample #1 comes from an animal that was hemizygous for the transgene. Sample #2 and 3 come from a single homozygous transgenic mouse milked on two separate occasions, one month apart. Control and transgenic milk samples #1 and 2 were frozen and thawed 2 times before assaying. Transgenic milk sample #3 was frozen and thawed only once before assaying. Each of the transgenic samples assayed above appear to have greater than 1 mg/ml of human urokinase activity in the undiluted milk.

(Fig. 3). We obtained similar results using a commercially available colorometric assay for plasminogen activator (data not shown). Non-transgenic control animals had no detectable plasminogen activator activity. That the activity observed in transgenic mouse milk was due to human urokinase, and not an endogenous plasminogen activator, was shown by duplicate activity assays in the presence of a monoclonal antibody that specifically blocks human urokinase activity (American Diagnostica Inc., New York, NY, catalog #394). This antibody also blocks the plasminogen activator activity in transgenic mouse milk (Fig. 3). The activity in mouse milk is associated with a protein that is the same size as authentic human urokinase as shown by combining the casein/plasminogen agarose gel assay with polyacrylamide gel electrophoresis (Fig. 4A). Western blot analysis confirmed that this activity is due to human urokinase (Fig. 4B). Further, a protein of the correct size in transgenic, but not in control, mouse milk is recognized by goat anti-human urokinase heteroserum.

DISCUSSION

The high level expression of human urokinase in the milk of transgenic animals does not appear to affect either the health of the mothers or the nursing pups. Transgenic mice give birth to multiple healthy litters of 8–10 offspring.

We have demonstrated that a heterologous protein can be produced at high concentrations, in an active form, in the milk of transgenic animals, and that regulatory sequences from the bovine α_{s1}-casein gene can be used to drive this high level expression. Because these bovine regulatory sequences function correctly in mice, we assume they should be at least as effective at directing the expression of heterologous genes in transgenic ruminants, and particularly in transgenic cows. If this expression system works as well in cows as it does in our mouse model, a single transgenic cow producing 5 gallons of milk a day could produce over 20 g of recombinant protein a day.

EXPERIMENTAL PROTOCOL

Gene constructs. Two cosmid clones, E1 and C9, were isolated from a cosmid library constructed in the vector pHC 79[10] using synthetic oligonucleotides based on published cDNA sequences. Cosmid E1 includes 21 kb of 5′ flanking sequence together with the first half of the α_{s1}-casein gene. Cosmid C9 extends from the second intron to 15 kb downstream of the polyadenylation site. An 8 kb BamHI fragment of C9 containing the polyadenylation site was subcloned and the 3′ BamHI site converted to a SalI site using the synthetic oligonucleotide adapter pGATCGTCGAC. The resulting plasmid, pCAS1238, was used to provide the 3′

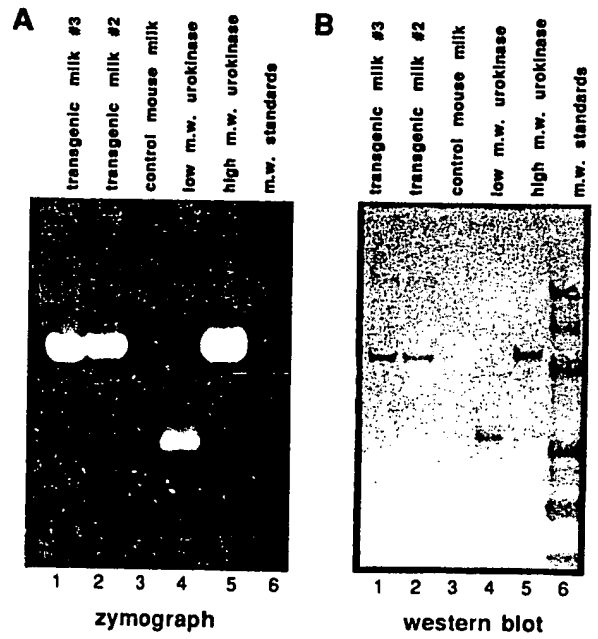

FIGURE 4 Electrophoretic analysis of transgenic mouse milk. The samples were diluted into non-reducing loading buffer (10% glycerol, 20 mM Tris pH 7.4, 3% SDS) and electrophoresed through duplicate 10–20% polyacrylamide gradient gels under nonreducing conditions. Lane 1, 0.15 μl milk from homozygous transgenic mouse (sample was frozen and thawed once); lane 2, 0.15 ml milk from same homozygous transgenic mouse (sample was frozen and thawed twice); lane 3, 0.15 μl milk from control nontransgenic mouse, frozen and thawed twice; lane 4, 100 ng low molecular weight human urokinase (#124. American Diagnostica, Inc. New York, NY); lane 5, 100 ng high molecular weight human urokinase (#125. American Diagnostica, Inc. New York, NY); lane 6, molecular weight standards: myosin (H-chain), phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, β-lactoglbulin, lysozyme (Bethesda Research Laboratories, Bethesda, Md.). (A) Zymograph of plasminogen activator activity. One of the gels was soaked in 2.5% Triton X-100 for 1 hr. and rinsed in water to remove the SDS. It was then placed on a casein agarose gel (described in the legend to Fig. 3) and incubated for 6 hrs. at 37°C, followed by staining with amido black. Zones of plasminogen activator activity appear white on a black background. (B) Western blot analysis. The second gel was electroblotted onto nitrocellulose membrane and probed with goat anti-human urokinase (#398, American Diagnostica, Inc. New York, NY), followed by incubation with horseradish peroxidase conjugated swine anti-goat immunoglobulin.

genomic sequences for the final construct. To provide 5′ sequences, a 4 kb SmaI/BamHI fragment of E1 including the transcription and translation start sites was subcloned. A BamHI site was introduced at the BglII site located 13 nucleotides upstream of the initiator methionine codon by inserting the synthetic oligonucleotide pGATCTTGGATCGAA. The resulting SmaI/BamHI fragment containing the transcription start site was subcloned together with the 8 kb BamHI/SalI insert of pCAS1238. The unique BamHI site in this plasmid was then replaced by XhoI and NotI sites using the synthetic oligonucleotide adapter pGATCTCGAGCGCGGCCGCGCTCCA. The resulting plasmid, pCAS1277, thus contains the α_{s1}-casein transcription start site fused to downstream genomic sequences including the polyadenylation site. The XhoI and NotI sequences bridging the 5′ and 3′ regions of the gene provide sites for introducing new coding sequences. The XmaI/SalI insert of this plasmid was cloned back into XmaI partially digested, SalI cut cosmid E1 to produce pCAS1288, which includes 20 kb of 5′ flanking sequences not found in pCAS1277. The human urokinase gene was isolated from a cosmid library[11] using synthetic oligonucleotides based on published sequences[12]. A 7.5 kb ApaI fragment containing the entire coding portion of the gene, together with 31 nucleotides of upstream and 1 kb of downstream sequences, was subcloned from one of the cosmid clones using a synthetic oligonucleotide adapter (pCGTCGACG/pGTACCGTCGACGGGCC) containing a SalI site. The resulting 7.5 kb SalI fragment was ligated to XhoI partially digested pCAS1288 to produce the hybrid casein/urokinase plasmid pCAS1295.

RNA analysis. Total RNA was isolated from fresh tissue using the acid guanidinium thiocyanate-phenol-chloroform extraction procedure described by Chomczynski and Sacchi[13]. Ten micrograms of RNA from each tissue was electrophoresed through a 1.1% agarose/2.2% formaldehyde gel in 50 mM HEPES, pH 7.2; 5 mM NaOAc; 1 mM Na_2EDTA. The gel was blotted onto an unmodified nylon membrane (GeneScreen, NEN) and cross-linked with UV light[14]. The membrane was then hybridized with a nick-translated cDNA clone—pUKO321 (provided by Kathy Hession, Biogen Inc.), encoding human urokinase—in 33% formamide, 9.3% SDS, 53 mM Na_2HPO_4, 9.3 mM Na_4EDTA, pH 7.2, at 55°C, and washed at 65°C in 7% SDS, 40mM Na_2HPO_4, 7 mM Na_4EDTA, pH 7.2, followed by 1% SDS, 6mM Na_2HPO_4, 1 mM Na_4EDTA, pH 7.2.

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Expression of active recombinant human α1-antitrypsin in transgenic rabbits

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Summary

A DNA construct containing the human α1-antitrypsin gene including 1.5 and 4 kb of 5′ and 3′ flanking sequences, was microinjected into the pronucleus of rabbit embryos. The recombinant human protein was (a) expressed in the blood circulation of F0 and F1 transgenic rabbits at an average concentration of 1 mg ml^{-1}, (b) shown to be fully active and (c) shown to be separable from its rabbit counterpart. Transgenic rabbits might represent a novel source of human proteins of therapeutic interest.

Human α1-antitrypsin; Transgenic rabbits; Purification from blood

Introduction

α1-Antitrypsin (α1AT) is a member of a family of serum protease inhibitors (Crystal, 1989). It is present at a concentration of several mg ml^{-1} in plasma and its major function is to deter neutrophil elastase activity in the lung (Carrell et al., 1982). The inhibitory specificity is determined by the presence of a few amino acids which form the active site of the molecule. Specific mutations in the gene lead to a hereditary deficiency state which predisposes the affected individuals to pulmonary

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emphysema (Gadek and Crystal, 1982; Janoff, 1985). Large quantities of recombinant (r) α1AT have been produced using *Escherichia coli* or yeast as cellular hosts and the therapeutic efficiency of these preparations is under assessment (Courtney et al., 1984; Casolaro et al., 1987; Wewers et al., 1987; Hubbard et al., 1988, 1989).

The cDNA of α1AT has been manipulated in order to generate novel molecules with amino acid modifications in the active site of the protein (Courtney et al., 1985). These engineered α1AT mutants display inhibitory activity against new substrates (thrombin and kallikrein, for instance) and could represent potentially new therapeutics for the treatment of diseases other than lung emphysema.

α1AT is a glycoprotein which is mainly synthesised in liver and is secreted in the blood circulation. Although the recombinant protein expressed in *E. coli* and yeast is biologically active, the absence of sugar side chains results in a reduced in vivo half-life, when compared to the plasma-derived counterpart (Casolaro et al., 1987; Hubbard et al., 1988).

For this reason, mammalian cells may represent an alternative candidate for the expression of active glycosylated human α1AT. Human α1AT has been expressed in the mammary gland of transgenic sheep (Simons et al., 1988). Expression levels were, however, too low to envisage transgenic sheep milk as an abundant source of rα1AT. On the other hand transgenic mice have been shown to express physiological levels (mg ml^{-1}) of human α1AT in their plasma (Kelsey et al., 1987; Sifers et al., 1987; Rüther et al., 1987; Dalemans et al., 1990). A recent report (Archibald et al., 1990) also describes the expression of biologically active human α1AT in the milk of transgenic mice.

In this paper we have investigated the possibility of generating transgenic rabbits designed to express high levels of active human rα1AT. Due to their high prolificity as well as convenient volumes of blood and milk, rabbits could represent an alternative transgenic host for the production of recombinant human proteins.

Materials and Methods

DNA microinjection

Rabbit adult females were superovulated by firstly injecting 0.25 mg of porcine FSH for 3 d, then 0.625 mg of porcine FSH for 2 d and eventually 0.3 mg of porcine LH. Mating was done on the day of LH injection at 4 p.m. Embryos were collected the next day at 9 a.m. after killing the animals. About 2 pl of DNA solution containing 500 copies of the gene construct previously described (Dalemans et al., 1990) were injected into the male pronuclei. The injected embryos were transferred to pseudo-pregnant females.

Detection of the transgene

Blood samples were collected from rabbits and directly diluted in 10 volumes of 10 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 1% 100 × Triton, 0.32 M sucrose (Ciulla et

al., 1988). Briefly, DNA from white cells was extracted with 4 M guanidinium isothiocyanate. Following isopropanol precipitation, the pellet was washed in 70% ethanol and treated with 100 mg ml^{-1} proteinase K overnight at 37°C in 0.5% sodium dodecyl sulfate (SDS). DNA was isolated after phenol/chloroform extraction and isopropanol precipitation.

Southern blotting was carried out by conventional methods. The human α1AT gene or cDNA cross-hybridizes with its rabbit counterpart. Cleavage of the human gene by *Pst*I generated several bands. Four of them (ranging from 0.6 to 0.7 kb) proved not to cross-hybridize with the rabbit α1AT gene. The doublet at 0.7 kb was extracted from an agarose gel and used as a probe. Filters were hybridized overnight at 65°C with randomly primed ^{32}P-labelled in 310 mM NaCl, 20 mM sodium phosphate (pH 7.0), 20 mM EDTA and in the presence of 2.5% dry, defatted milk (Johnson et al., 1984). The filters were extensively rinsed in 4 × SSC and further washed for 30 min in 0.1 × SSC/1% SDS at 65°C.

Northern blot

Total RNA from various tissues was extracted in the presence of guanidinium isothiocyanate and phenol (Puissant and Houdebine, 1990). After agarose gel electrophoresis, RNA was transferred to Zeta-probe filters and fixed by UV irradiation (Khandjian, 1986). Hybridization was carried out overnight at 65°C with the whole human α1AT cDNA as probe in 0.5 M sodium phosphate buffer (Mahmoudi and Lin, 1989) in the presence of 0.5% dry, defatted milk. Filters were washed extensively in 4 × SSC at 65°C and subsequently autoradiographed.

Detection of human α1AT in rabbit plasma

α1AT antigen levels were monitored by ELISA (Gilardi et al., 1990).

Purification of human α1AT from rabbit plasma

Polyclonal antibodies were purified from goat polyclonal antiserum against human α1AT (Organon Teknika, West Chester, PA, U.S.A.), that did not cross-react with rabbit α1AT by ammonium sulfate precipitation (50% saturation) followed by ion-exchange HPLC on a Baker Abx column as described by the manufacturer (Baker, Gaithersburg, MD, U.S.A.).

The purified antibodies were subsequently immobilized on cyanogen bromide activated Sepharose 4B® (Pharmacia, Uppsala, Sweden) according to the manufacturer's protocol to a concentration of 5 mg g^{-1} of gel.

Elution conditions for human α1AT were screened by ELISA coating the antigen on the plate and incubating it with the purified antibodies, as proposed by Dr. Claus Koch (Statens Serum Institute, Copenhagen, Denmark). After washing the plate with a range of different potential eluents the ELISA was developed using biotinylated anti-goat polyclonal antibodies. Among 12 tested eluents only 0.1 M sodium citrate (adjusted to pH 2.8 with phosphoric acid) proved to be effective.

Unfortunately human α1AT rapidly lost its inhibitory activity under these conditions.

Immunoaffinity chromatography was done by loading 300 μl of rabbit plasma (transgenic or normal) onto a 1 ml column equilibrated in 20 mM sodium phosphate (pH 7.4). The support was subsequently washed with 5 column volumes of 20 mM sodium phosphate (pH 7.4) containing 1 M NaCl (to remove rabbit α1AT) and finally 0.1 M sodium citrate, (pH 2.8). Most human α1AT was eluted in the first 5 ml. The eluted α1AT was concentrated in a diafiltration unit (10 kDa cut-off; Amicon, Danvers, MA, U.S.A.) and precipitated with acetone for analysis by polyacrylamide gel-electrophoresis (10% acrylamide) under denaturing and reducing conditions.

Anti-elastase activity

Elastase inhibitory activity in rabbit plasma was quantitated by titrating a known amount of human neutrophil elastase (Elastin Products, Owensville, MO, U.S.A.) with increasing amounts of plasma. Residual elastase activity was measured after a pre-incubation time of 5 min at room temperature by adding the incubation mixture to a buffered solution containing 2 mM of the chromogenic substrate methoxy-L-alanyl-L-prolyl-L-valine-*p*-nitroanilide (Calbiochem, La Jolla, CA, U.S.A.) and measuring the absorbance change at 410 nm at 37°C over a time period of 3 min. Graphical analysis of the rate of substrate hydrolysis plotted against the amount of added plasma by linear regression gave the concentration of active inhibitor. In order to inhibit human α1AT in the plasma of transgenic rabbits a pre-incubation with specific purified polyclonal antibodies (0.125 mg ml^{-1}) for 1 h at room temperature preceded the titration of neutrophil elastase.

Results

Gene construct

The transgene construct used for microinjection has been described (Dalemans et al., 1990). It consists of the complete human α1AT gene including 1.5 kb of 5′ and 4 kb of 3′ flanking sequences, respectively. An additional expression block in which the human α1AT promoter drives the expression of the murine c-*myc* gene is also present.

Generation of transgenic rabbits

Seven hundred and fifty-six microinjected embryos were transferred into 41 pseudopregnant females, of which 13 did not become pregnant. One hundred and nine rabbits were born (about 13% of the transferred embryos) and 77 of them, which survived after weaning, were tested for the presence of the transgene. One founder rabbit (termed the progenitor) was positive on Southern blotting (Fig. 1).

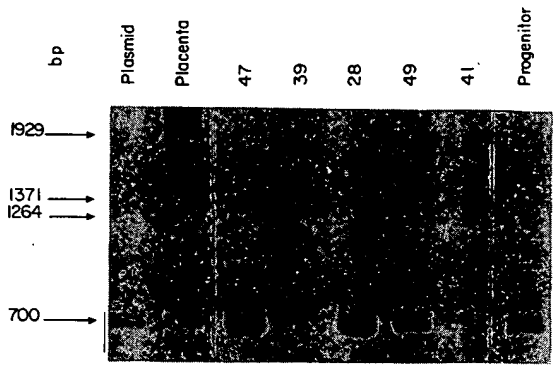

Fig. 1. Southern blot of genomic DNA from progenitor and several F1 rabbits. DNA was extracted from blood cells digested with *Pst*I, subjected to Southern blotting and probed with the 0.7 kb fragments from the human α1AT gene as described in Materials and Methods. Plasmid: *Pst*I restricted plasmid containing the human gene. Placenta: 10 μg of genomic DNA from human placenta. Numbers indicate F1 rabbits derived from the progenitor. Rabbits 39 and 41 showed no signal and no human α1AT expression in blood.

Two bands of about 700 bp recognized by the probe used (see Materials and Methods) were present in the DNA from the progenitor rabbit and from human placenta. As judged by the intensity of the band in the autoradiogram, the number of transgene copies was 5.

The progenitor was mated with normal females. About 8 offspring were recovered per pregnancy. Among the 132 F1 animals, 28 contained the transgene (Fig. 1). This resulted in 21% of the offspring being transgenic, a fact which suggested that the progenitor was mosaic. The specific bands at 0.7 kb were found in the offspring as in the progenitor indicating that the transgene was transmitted essentially without rearrangement. The intensity of the bands was higher in the offspring than in the progenitor, thus suggesting that more copies of the transgene were present in the genome of the offspring and confirming the mosaicism of the progenitor.

Expression of the transgene

Human α1-antitrypsin antigen levels were measured in plasma of F0 and F1 rabbits. The results summarized in Fig. 2 indicate that the progenitor and offspring expressed human α1AT antigen at an average concentration of about 1 mg ml^{-1}. Consecutive measurements carried out on different blood samples from the same animals at different times showed slight variations.

Northern blot analysis (Fig. 3) revealed that the transgene was expressed essentially in liver and in kidney, no signal being detected with RNA from muscle, spleen, pancreas and (not shown) intestine, testis, brain and adrenal gland. A major band migrating at the expected site for the α1AT mRNA was detected and was consistent with correct splicing of the mRNA. RNA from normal rabbit liver

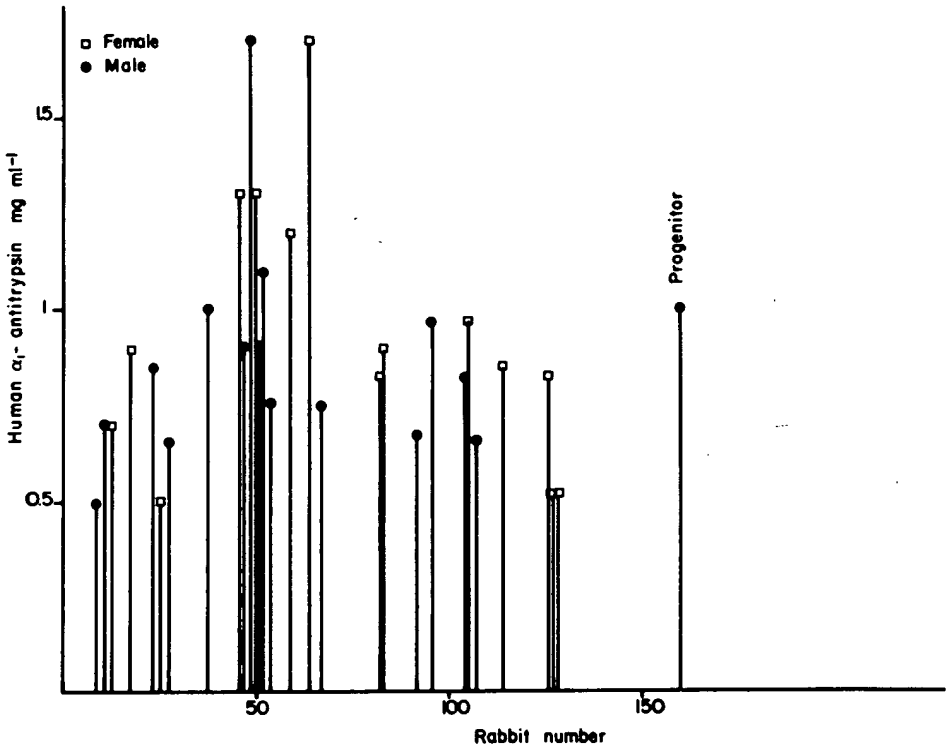

Fig. 2. Antigen concentration of human α1AT in plasma of the transgenic progenitor and of its offspring as detected by ELISA. Negative rabbits gave negative signals by slot blotting and (rabbits 39 and 41) by Southern blotting.

showed a slight but significant cross-hybridization with the human cDNA probe. Additional bands of larger size were also observed in the RNA from the transgene rabbit liver.

Purification and characterization of human α1AT from rabbit plasma

Human α1AT was isolated from the pooled plasma of F1 transgenic rabbit by immunoaffinity chromatography using purified polyclonal antibodies which did not cross-react with rabbit α1AT. The purified rα1AT migrated similarly to purified α1AT from human plasma on polyacrylamide gels, after electrophoresis under denaturing and reducing conditions (Fig. 4, lanes 3, 5). Non-glycosylated rα1AT isolated from *E. coli* showed a significantly reduced apparent molecular weight (Fig. 4, lane 2). Rabbit α1AT was not detected in normal rabbit plasma immunopurified by the same procedure (Fig. 4, lane 4) proving that the immunoisolation was specific for human α1AT. N-terminal aa analysis (21 residues) of the isolated α1AT from transgenic rabbits shows correct processing of the human rα1AT.

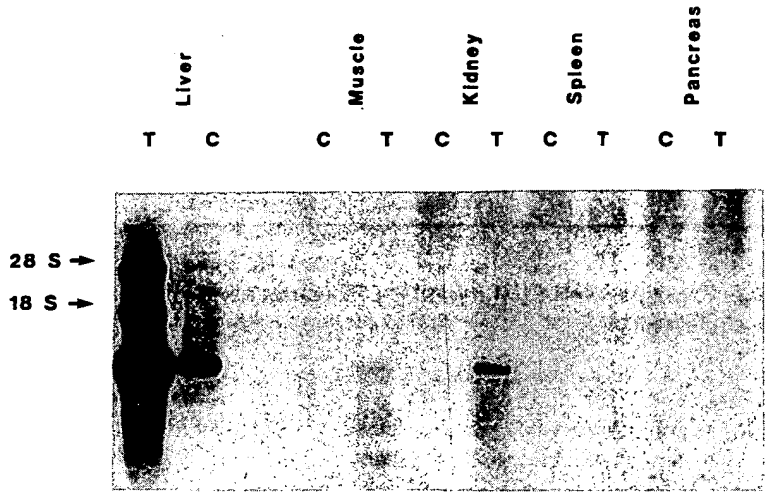

Fig. 3. 10 μg of total RNA were electrophoresed on agarose gel. The probe used was the entire human α1AT cDNA. Autoradiography lasted one week. Positions of 18 S and 28 S RNA are indicated by arrows. T: transgenic; C: control.

In order to evaluate whether the expressed human α1AT was active as an inhibitor, human neutrophil elastase was incubated with plasma from transgenic and normal rabbits in the presence and absence of specific polyclonal antibodies neutralizing (80%) the inhibitory activity of human plasma-derived α1AT under the employed conditions. Table 1 shows that the human α1AT activity neutralized by the antibodies corresponds to a calculated value of 1.09 mg ml^{-1} which is similar to

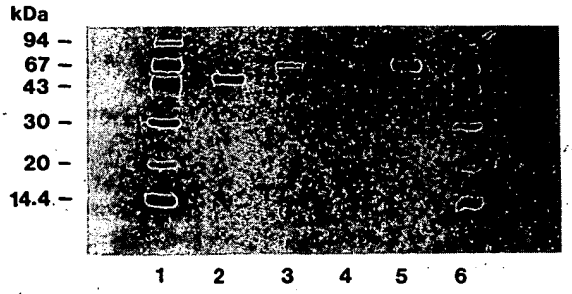

Fig. 4. SDS-PAGE (10%) of immunopurified samples and controls under denaturing and reducing conditions. Lanes 1 and 6: molecular weight standards. Lane 2: recombinant α1AT isolated from *E. coli*. Lane 3: α1AT isolated from the plasma of transgenic rabbits. Lane 4: control (normal rabbit). Lane 5: α1AT isolated from human plasma.

TABLE 1

Anti-elastase activity in rabbit plasma

Active inhibitor (mg ml^{-1})	Rabbit plasma
1.32	Normal
1.37	Normal + neutralizing antibodies
2.45	Transgenic
1.36	Transgenic + neutralizing antibodies

the measured antigen levels (0.94 mg ml^{-1}). Thus transgenic α1AT is fully active as an elastase inhibitor.

Discussion

The data reported here confirm previous work (Hammer et al., 1986; Knight et al., 1988; Bühler et al., 1990) showing that the technology for generating transgenic rabbits is available. The proportion of transgenic births was in all cases lower than that generally observed in the case of transgenic mice. This may be due to the fact that DNA synthesis is already initiated when microinjection was performed (Oprescu and Thibault, 1965). In fact, Hammer et al. (1986) reported that a higher proportion of transgenic rabbits could be obtained when microinjections were carried out soon after fertilization. Under the experimental conditions of the present work, the foreign DNA may not have been integrated until after the first division leading to a reduced number of transgenic animals and to mosaicism.

The foreign gene was transmitted to progeny without detectable rearrangements. The mRNA coding for the human α1AT was found essentially in liver and to a lower degree in kidney of transgenic rabbits. This observation is similar to results obtained in transgenic mice (Kelsey et al., 1987; Sifers et al., 1987, 1989; Rüther et al., 1987; Carlson et al., 1988; Koopman et al., 1989; Shen et al., 1989; Sepulveda et al., 1989; Dalemans et al., 1990). Endogenous α1AT gene expression in humans was found in other tissues such as stomach, pancreas, salivary glands, etc. (Carlson et al., 1988). However, such mRNA transcripts were observed using in situ hybridization or S1 nuclease protection assay. In transgenic rabbits the sensitivity of the Northern blot technique may have been too low to allow detection of the human α1AT in other tissues than liver and kidney.

Expression of the human α1AT in blood was high and in the same order of magnitude of that observed in humans and in transgenic mice (Sifers et al., 1987, 1989; Rüther et al., 1987). The capability to express the human α1AT gene at a high level was transmitted to progeny.

The gene construct used in the present work contained in addition the murine c-*myc* proto-oncogene under the control of the human α1AT promoter. The presence of this additional gene in the construct is not likely to interfere significantly with the expression of the human α1AT gene since the same gene without the c-*myc*

gene is similarly efficient in transgenic mice (Kelsey et al., 1987; Sifers et al., 1987; Rüther et al., 1987).

In this paper we have demonstrated that transgenic rabbits can be used as a potential source of human proteins of therapeutic interest. Moreover, the fact that these rabbits express the murine c-*myc* in a liver-specific manner and that some of them develop hepatomas consisting of isolated nodules of malignant cells (preliminary results), could potentially lead to the establishment of trans-immortalized hepatic rabbit cell lines secreting human α1AT as previously demonstrated in the case of transgenic mouse lymphomas (Pavirani et al., 1989) and hepatomas (Dalemans et al., 1990; Jallat et al., 1990).

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TRANSGENIC PRODUCTION OF A VARIANT OF HUMAN TISSUE-TYPE PLASMINOGEN ACTIVATOR IN GOAT MILK. GENERATION OF TRANSGENIC GOATS AND ANALYSIS OF EXPRESSION

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We report the first successful production of transgenic goats that express a heterologous protein in their milk. The production of a glycosylation variant of human tPA (LAtPA - longer acting tissue plasminogen activator) from an expression vector containing the murine whey acid promoter (WAP) operatively linked to the cDNA of a modified version of human tPA was examined in transgenic dairy goats. Two transgenic goats were identified from 29 animals born. The first animal, a female, was mated and allowed to carry the pregnancy to term. Milk was obtained upon parturition and was shown to contain enzymatically active LAtPA at a concentration of 3 μg/ml.

Several types of human proteins have been expressed in lactating mammary glands of transgenic mice and the proteins secreted into milk (reviewed in refs. 1,2). The scale-up of mouse model systems to livestock species may ultimately provide an alternative production system to the commonly used mammalian tissue culture production processes. A transgenic mammary gland production system would have the potential advantages of not requiring an intensive capital expenditure in setting up a manufacturing facility, and of providing a highly cost efficient system due to high expression levels and low expendable production costs.

Most of the work to date has been done in the mouse model system. Regulatory sequences of the whey acid protein (WAP), β- and α-lactoglobin (BLG) and β-casein genes have been used to target expression of numerous genes to the lactating mammary gland, including tissue plasminogen activator (tPA)[3,4], human anti-hemophilic factor IX[5], soluble CD4[6], human interleukin-2[7], and human α_1-antitrypsin[8]. A vector system based on the αS1-casein gene has also been tested in a mouse model where human urokinase was produced efficiently in milk at levels 10 times greater than those achieved in cellular expression systems[9].

Whereas model systems have demonstrated the feasibility of targeting gene expression to the lactating mammary gland and secretion of heterologous proteins into milk, efficient generation of transgenic livestock and production of foreign proteins in their milk have proven more difficult to achieve. Early work aimed at the generation of transgenic farm animals led to low frequencies of integration, low number of animals that expressed the recombinant proteins, reproductive problems, and resultant physiological problems[2]. By targeting protein synthesis to an exocrine organ in which expressed proteins would be expected to be sequestered away from the circulation and removed from the animal, we and others hope to bypass some of these potential problems in the generation of transgenic livestock. In fact, transgenic sheep, which produce factor IX in their milk, have been generated and apparently exhibit no physiological or reproductive problems[5], although expression levels in these animals were low. More recently, however, high expression of the murine whey acidic protein in transgenic swine and mice may have had adverse effects on the physiology of the mammary gland[10].

We have aimed to produce a commercial prototype for the large-scale manufacture of high market-volume proteins in the transgenic mammary gland system using the dairy goat as a production animal. The goat was chosen for several reasons: (1) Dairy goats produce large volumes of milk, on average 4 liters per day; (2) goats have gestation and development periods of moderate length (5 and 8 months respectively); and (3) goat milk has been extensively characterized at the biochemical level[11]. In this paper, we describe the first successful generation of transgenic goats at frequencies that approach those in the rodent systems. More importantly, a transgenic goat was generated that produced an enzymatically active form of tPA throughout a normal lactation period. These experiments further support the concept of targeting expression of transgenes that encode pharmaceutical proteins to the mammary gland of dairy livestock.

RESULTS AND DISCUSSION

Generation of transgenic goats. The expression vector WAP-tPA was generated previously by fusing a 2.6 kb EcoRI-KpnI fragment upstream of the murine whey acid protein gene to a cDNA encoding wild type human tPA[3]. This vector led to expression of tPA in milk of transgenic mice at levels as high as 250 μg/ml (data not shown). A structural tPA variant was constructed (designated LAtPA) in which an asparagine to glutamine point mutation was introduced into the cDNA to produce a recombinant protein devoid of glycosylation at residue Asn 117. This longer acting tPA variant had an increased systemic half-life in a rabbit model[12]. A DNA fragment containing this point mutation in the tPA cDNA was substituted for the equivalent fragment in WAP-tPA to generate the vector used in this study, WAP-LAtPA (Fig. 1).

Goat embryos were flushed surgically from the oviducts of superovulated dairy goats as described in the Experimental Protocol. The superovulation protocol had been

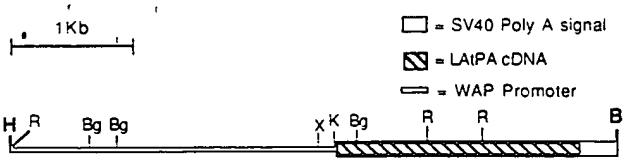

FIGURE 1 A schematic representation of the WAP-LAtPA expression vector and its restriction enzyme sites used for the production of transgenic goats. H = HindIII, R = EcoRI, Bg = BglII, X = XbaI, B = BamHI, K = KpnI.

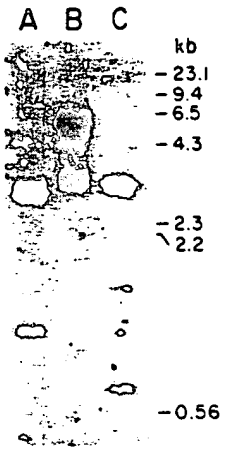

FIGURE 2 Southern blot hybridization of transgenic goat DNA. DNA isolated from the blood of goat #1 was digested, fractionated on an agarose gel, and hybridized to a probe from the whey acid protein gene as described in the Experimental Protocol. Hybridization of this probe to DNA from non-transgenic animals gave no signal (data not shown). Lanes A: BglII; B: XbaI; C: EcoRI.

previously optimized to result in the highest yield and proportion of one-cell embryos[13]. In the course of this study, a total of 372 embryos or ova were collected from 63 donor animals. Of the embryos collected, 252 (68%) were zygotes, 24 (7%) were 2-cells, 5 (1%) were 4-cells and 91 (25%) were unfertilized as determined by the absence of pronuclei. Twenty-eight percent of the injected fertilized embryos were considered poor injections due to the non-optimal positioning of the injected pronuclei, i.e., the pronuclei were at the periphery of the cell making the nuclear injection very difficult. Approximately 8% of the fertilized embryos collected had to be centrifuged for 30 seconds at 13,000 × g to visualize the pronuclei under Normarski optics. Due to the limited number of recipients and donors available on any given experimental day, microinjected 1-cell, centrifuged 1-cell, and 2-cell goat embryos were typically mixed prior to transfer to the recipient females. This prevented us from confirming the viability of the centrifuged eggs or the potential production of transgenic goats by microinjection of 2-cell embryos. However, subsequent experiments with other fusion genes have confirmed that microinjected centrifuged 1-cell goat embryos are viable and can produce viable offspring. In addition, we have also produced a transgenic goat (potentially a mosaic) that was the result of injection of a 2-cell goat embryo (data not shown).

Embryos were injected with a 4.9 kb HindIII-BamHI fragment of WAP-LAtPA purified free of procaryotic DNA (Fig. 1) at a concentration of 1 μg/ml in 10 mM Tris, pH 7.5, 0.1 mM EDTA, and either immediately transferred to the oviducts of recipient females or cultured in Ham's F12 medium containing 10% fetal calf serum in an atmosphere of 5% CO_2 in air at 37°C for 72 hours and subsequently transferred to the uterus of recipient females. Typically the cultured goat embryos were blocked at the 8-16 cell stage in this culture system, but remained viable and could produce live offspring (Table 1).

Pregnancies were confirmed by the inability of recipient animals to return to natural estrus and by ultrasonic examination on days 45 and 55 of pregnancy. Ultrasound on days 45 and 55 of pregnancy have resulted in a 100% early pregnancy confirmation over the first three years of this project. A complete summary of the recipient data is shown in Table 1. Twenty-nine animals were born from 203 embryos transferred, representing 14% of injected embryos surviving to term. Samples of blood and ear tissue from each goat were analyzed by Southern blotting in order to detect transgenics and to confirm that mosaic animals did not escape detection. Two animals were identified as transgenic; a female, goat #1, and a male, goat #21. The generation of 2 transgenic animals in 29 goats born from injected embryos represents an integration rate of 7% and can be compared to frequencies previously reported for mouse (10–30%[14]), rabbit (9.5%[7]), pig (10–40%[15]) and sheep (6–10%[16,5]).

At nine months of age, goat #1 was mated to a non-transgenic male. She became pregnant without difficulty and delivered two non-transgenic progeny. A second pregnancy resulted in three additional offspring, one of which was transgenic. Goat #21 (male) was aspermic and was subsequently diagnosed to have a bilateral spermatocele at the head of the epididymis resulting in blockage of normal, developed sperm from entering the vas deferens;

TABLE 1 Summary of injected goat embryos transferred to recipient females.

A.

No. Recipients	Centrifugation	Transfer To	No. Embryos/Stage	No. Pregnant	No. Offspring	No. Transgenic
28	No	Oviduct	137/1-cell 18/2-cell	13[a]	22	2
2	Yes	Oviduct	10/1-cell	0[a]	0	0
5	No	Uterus	31/morula[b]	4	5	0
1	Yes	Uterus	7/morula[b]	1	2	0

B. Efficiency

No. Recipients	No. Embryos	No. Pregnant (%)	No. Offspring (%)	No. Transgenic (%)
36	203	18 (50.0)[c]	29 (14.3)[d]	2 (6.9)[e]

[a]Two of the pregnant animals aborted prematurely.
[b]Embryos injected at the 1-cell stage and cultured for 72 hours.
[c]Percentage is the number of pregnant animals per number of recipients.
[d]Percentage is the number of offspring born per number of embryos transferred.
[e]Percentage is the number of transgenic animals per live births.

a probable congenital defect that is commonly seen in goats. Therefore, we are unclear as to whether this defect relates to the integration of the transgene.

In order to determine the arrangement of the transgene and to estimate the number of copies per cell, blood DNA from the founder female (#1) was digested with three endonucleases and analyzed by Southern blot (Fig. 2). The blot was probed with a 580 bp EcoRI-BglII fragment from the 5′ end of the WAP upstream region labeled to a specific activity of 1×10^8 cpm/μg of DNA by the random hexamer labeling technique[17]. BglII digestion (Lane A) resulted in a predictable 2.9 kb fragment and a 5′ junction fragment approximately 1.0 kb. BglII is known to cut at three sites within the fusion gene (Fig. 1). The lower intensity of the junction fragment indicates that the integrant was inserted at one site and had multiple copies. XbaI (Lane B) cuts once within the fusion gene resulting in a predictable 4.9 kb fragment if the fusion gene integrated in multiple copies and in a tandem array in normal orientation, as well as 5′ junction fragment of approximately 3.3 kb. EcoRI cuts the fusion gene into three fragments (Fig. 1) of which the 3.3 kb fragment will hybridize to the probe on the Southern blot (Lane C).

Figure 3 shows a Southern blot of an EcoRI digest of DNA extracted from blood cells from goat #1 and her five offspring. The blot was probed with a 1.7 kb LAtPA cDNA fragment. The Southern blot shows 3 major bands representing 3.3 kb, 1.1 kb and 472 bp fragments that corresponds to the 5′, 3′, and interior components of the fusion gene respectively. A minor band of 1.6 kb represents the 3′ junction fragment. The probe is weakly homologous to goat DNA. The initial restriction pattern of genomic DNA from the founder female was consistent with the presence of one to two integrated copies of the tPA transgene. However, as shown in Figure 3, the transgenic offspring was shown to contain more copies of the transgene per cell than the founder. This can be interpreted to mean that the founder animal was a mosaic with more copies of the fusion gene per cell than was originally estimated. Additionally, when the blots were counted on a Beta Scope, the data indicated that the bands in offspring 1-3 were twice the value of the #1 founder and supports the concept that the female founder was a mosaic (data not shown). It is estimated from the Southern analysis that the transgenic offspring contained approximately 3-5 copies of the transgene per cell. It should be noted that Southern analysis of DNA from ear tissue gave identical information.

Expression of tPA in milk. The transgenic mother was milked manually twice per day with an average daily yield of 3-4 liters. Milk was stored frozen at −20°C and thawed just prior to analyses. ELISA and amidolytic assays were run on representative milk samples from the first two months of lactation with continued ELISA assay up to the end of lactation (240 days). The daily volume and concentration of LAtPA is shown in Figure 4A. The animal expressed LAtPA at approximately 3 μg/ml during the peak lactation period (1 to 140 days) with an increase in concentration (6.0 μg/ml) toward the end of the lactation period (141 to 240 days). The second lactation produced LAtPA at the same concentration as the first lactation. The first lactation period was truncated from a normal 300 days to 240 days to eliminate the hand milking procedure. The daily milk output and lactation curve were characteristic of a normal dairy goat during her first lactation. The apparent rise in output of LAtPA during the later part of the lactation period did not parallel the constant total protein concentration in the same milk samples. The elevated milk production from September 17 to November 12 corresponds to the initiation of her second estrus season (first

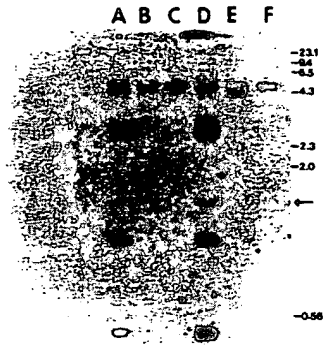

FIGURE 3 Southern blot hybridization of the founder transgenic goat #1 (Lane A) and her five kids (Lanes B, C, D, E, F). DNA isolated from blood was digested with EcoRI, fractionated on an agarose gel, and hybridized to a 1.7 kb LAtPA cDNA fragment. Hybridization of this probe to the transgene shows 3 bands: A: 3.3 kb 5′ junction fragment (Band 1); a 1.1 kb 3′ fragment (Band 2); a 472 bp interior fragment (Band 3). A minor band of 1.6 kb represents the 3′ junction fragment (arrow). Kid #1-3 (Lane D) was shown to be transgenic. Note the intensity of the bands from kid #1-3 was twice as intense as the founder female #1 and indicates that the founder animal is probably a mosaic.

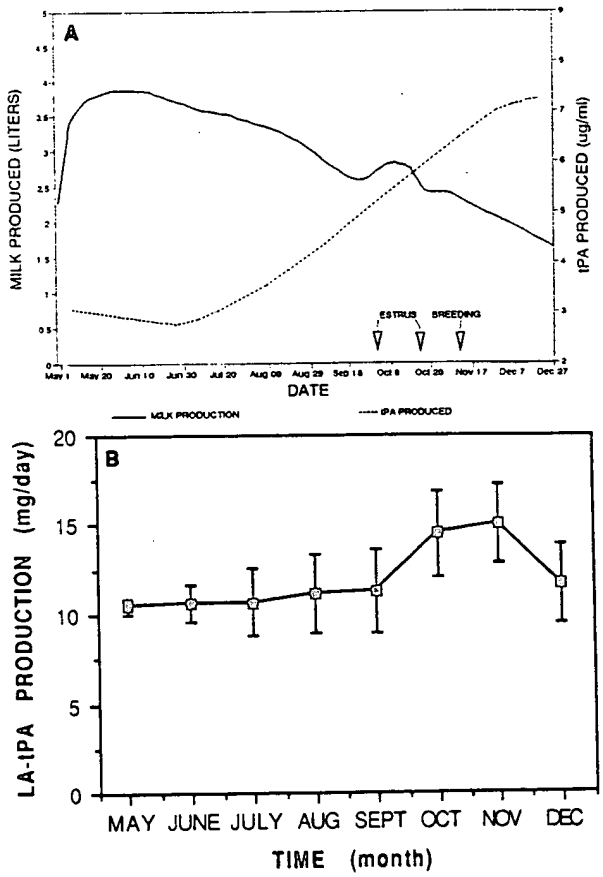

FIGURE 4 (A) LAtPA production throughout lactation in a transgenic goat. The solid line represents the best fit line for daily milk production throughout the 240 day lactation period. The dotted line represents the best fit line for the daily concentration of LAtPA in the milk. The days of her first, second and third estrus cycle and breeding are indicated on the graph. (B) Stability of expression of LAtPA in the milk of a transgenic goat (#1). Milk was harvested and stored as described in the accompanying paper. The concentration of the recombinant enzyme in the milk was determined by ELISA. Total LAtPA produced per day was calculated and daily averages and standard deviations for individual months determined by pooling the data from seven days during the course of each month.

estrus = October 3; second estrus = October 24; third estrus = November 12). The total amount of LAtPA produced per day was calculated and daily averages and standard deviations for individual months are shown in Figure 4B. The output of LAtPA remained relatively constant during the course of lactation, ranging between 11 and 15 mg/day. Although the concentration of the enzyme in milk was relatively low, i.e., approximately 10% of that observed in the recombinant C127 cell line, the continual expression was encouraging since it implied consistent production can be achieved in transgenic animals. The slight rise in output of LAtPA during October and November corresponds to estrus activity. Although the basis for this increase is not known, it is possible that the hormonal changes in the animal associated with the breeding season could have some effect on the expression of the transgene. The female was successfully rebred on November 12 and 13. Interestingly, this period of estrus cyclicity corresponded to a significant rise in the total amount of LAtPA being produced per day. The LAtPA produced in the milk was enzymatically active at approximately 610,000 U/mg. A detailed characterization of the protein is reported in an accompanying paper (Denman et al., *Bio/Technology: This issue*).

These experiments show that targeting transgenes that code for medically important pharmaceutical proteins to the mammary gland of dairy goats is feasible. The level of LAtPA was not high (3 μg/ml) in this first transgenic goat. The likelihood that this goat is mosaic may not allow us to achieve the actual expression level of this gene construct until we generate an F_1 female. However, we have recently produced another female transgenic goat that is producing LAtPA from a β-casein promoter at 2-3 mg/ml (data not shown). At this concentration, the dairy goat may be an economically viable bioreactor for human pharmaceuticals.

EXPERIMENTAL PROTOCOL

Production of transgenic goats. Goats used as donor animals were of either Alpine or Saanen breeds. The timing of estrus was synchronized in the donors with norgestomet ear implants (Syncromate-B, CEVA Laboratories, Inc., Overland Park, KS; 6 mg). Prostaglandin was administered after the first 7-9 days to remove endogenous sources of progesterone. At day 13 following progesterone administration, follicle-stimulating hormone (FSH, Schering Corp., Kenilworth, NJ) was given to goats at a dose of 18 mg over three days in twice daily injections (Warren Foote, personal communication). During the anestrus season (after February), the dose of FSH was increased to 24 mg administered similarly over three days in twice daily injections. Twenty-four hours following implant removal, the donor animals were mated several times to fertile males over a two-day period. Recipient animals were synchronized by the same protocols as the donor animals except that a single non-superovulatory injection of pregnant mares serum gonadotropin (PMSG, Sigma, St. Louis, MO) was given on day 13 of progesterone treatment in place of the FSH. From September to January, the recipients received 400 IU PMSG, and from February to April they received 750 IU PMSG. Recipient females were mated to vasectomized males to ensure estrus synchrony. Seventy-two hours following implant removal, embryos were recovered surgically from the oviducts of donors. Embryos were flushed from oviducts associated with ovulated ovaries through a cannula with sterile phosphate-buffered saline and were collected in a petri dish as previously reported[13]. The HindIII-BamHI fragment of WAP-LAtPA was injected into one of the two pronuclei from one-cell embryos or into a nucleus of one blastomere of two-cell embryos at a concentration of 1 μg/ml. Embryos were surgically transferred into the oviducts of the recipient females or to the uteri following a 72 hour culture period.

Identification of transgenic goats. DNA was extracted from the buffy coat recovered from blood of goat #1. Following digestion with restriction enzymes as indicated in the legend to Figure 1, DNA was fractionated and blotted onto nitrocellulose[18]. The probe was a 1.7 kb LAtPA cDNA isolated from the region of the whey acid protein gene 2600 bp upstream of the transcriptional start site[3]. The probe was radioactively labeled by the random primer method[17].

Enzyme activity and protein assays. Plasminogen activator concentrations (amidolytic activity) were determined with an indirect method using the plasmin substrate Val-Leu-Lys-*p*-nitroanilide[12] (S-2251, Helena Labs, Inc.). LAtPA concentration was estimated using the Imubind® tPA ELISA assay kit (American Diagnostics, Chicago, IL) adapted to determine LAtPA in goat's milk.

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БИОХИМИЯ, БИОФИЗИКА, МОЛЕКУЛЯРНАЯ БИОЛОГИЯ

УДК 636.3:575.113:577.113

ПОЛУЧЕНИЕ ОВЕЦ, ТРАНСГЕННЫХ ПО ГЕННОЙ КОНСТРУКЦИИ αS1-КАЗЕИН/ХИМОЗИН

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Одним из перспективных направлений генетической инженерии является создание трансгенных животных – продуцентов биологически активных белков фармакологического и технологического назначений [1 - 3].

В плане реализации международной программы "Генные фермы" нами выполнен комплекс работ по получению первичных трансгенных овец – продуцентов с молоком белка химозина крупного рогатого скота, являющегося главным энзиматическим компонентом сычужного экстракта молочных телят, который используется в технологии производства сыра. В молекуле каппа-казеина химозин катализирует расщепление только одной пептидной связи, в результате чего происходит свертывание молока.

Ген прохимозина кодирует пептид длиной 365 аминокислот, а также сигнальный пептид, состоящий из 16 аминокислот, обеспечивающий транспорт протеина через мембрану клетки.

В кислой среде прохимозин автокаталитически превращается в активную форму (фермент химозин) путем отщепления пептида из 42 аминокислотных остатков с N-конца [4].

В экспериментах на овцах для обеспечения тканеспецифической экспрессии в молоко использовали конструкцию baS1Cas–Chym длиной около 14 кб, в которой ген прохимозина крупного рогатого скота был поставлен под контроль регуляторных элементов гена αS1-казеина крупного рогатого скота [5].

Работу по получению трансгенных овец проводили на чистопородных животных многоплодной романовской породы со спонтанной охотой. Животных в охоте выбирали два раза в день (утром и вечером). Через 12 ч после выявления охоты маток естественно случали или искусственно осеменяли. Спустя 28 - 30 ч зиготы извлекали хирургическим способом у наркотизированных доноров путем промывания яйцеводов со стороны воронки. Этот метод позволял получать от одной овцы в среднем 3.3 яйцеклетки, из которых около 70% имели визуализируемые пронуклеусы [6].

Зиготы помещали в каплю питательной среды H199 ("ICN Flow"), дополненной 0.33 мМ пирувата Na, 15% фетальной сыворотки коров ("Sigma"), 100 МЕ/мл сульфата стрептомицина и забуференную 20 мМ HEPES. Для микроманипуляций использовали микроскоп "Axiovert 35", снабженный интерференционно-контрастной оптикой Номарского, микроманипулятор KM-2 и микроинъектор Narishige. С помощью стеклянной пипетки в мужской пронуклеус зигот инъецировали 1 - 2 пкл раствора ДНК (1000 копий/пкл в буфере ТЕ: 10 мМ *трис*-HCl, 0.2 мМ EDTA, pH 7.4). Об успехе микроинъекции судили по увеличению объема пронуклеуса.

Для пересадки микроинъецированных зигот был применен конвейерный метод, состоящий в использовании доноров зигот в качестве реципиентов: зиготы, полученные от первого донора, после микроинъекции пересаживали второму донору; зиготы от второго донора пересаживали третьему донору и т.д. Последнюю трансплантацию осуществляли "чистому" реципиенту [7, 8]. Каждой овцематке в один или в два яйцевода (в зависимости от наличия желтых тел) пересаживали 2 - 4 микроинъецированные зиготы. Время с момента вымывания до пересадки зигот, включая микроинъекцию, не превышало 20 мин.

ДНК для определения трансгенности животных-трансплантантов выделяли из тканей уха по методике Blin и Stafford [9], модифицированной Ausubel et al. [10]. Концентрацию ДНК и степень ее очистки определяли на спектрофотометре [11]. Анализ интеграции рекомбинантной ДНК проводили методом полимеразной цепной реакции

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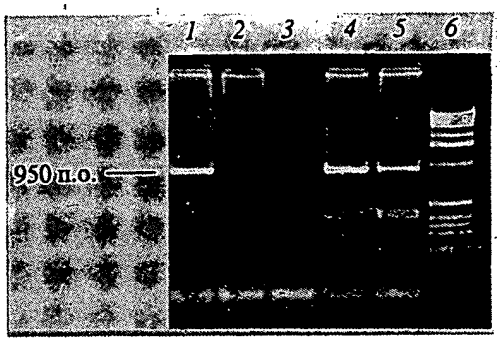

Рис. 1. Электрофореграмма PCR-анализа ДНК овец на наличие генной конструкции baS1Cas–Chym. *1* – ДНК баранчика № 1 (586-1, F1), *2* – ДНК баранчика № 2 (586-2, F1), *3* – контроль (все компоненты за исключением ДНК), *4* – ДНК овцы № 586 (FO), *5* – ДНК овцы № 596 (FO), *6* – маркер (1 kb ladder). Фрагмент ДНК длиной 950 п.н. соответствует амплифицированному фрагменту генной конструкции baS1Cas-Chim.

(PCR) [12]. С этой целью 50 - 100 нг геномной ДНК в 15 мкл реакционной смеси, содержащей 1× PCR-буфер (67 мМ *трис*-HCl, 16.6 мМ $(NH_4)_2SO_4$, 0.01% *твин*-20), 200 мкМ дНТФ ("Pharmacia"), 10 пМ каждого из праймеров и 0.7 Ед Taq-полимеразы ("Amersham"), подвергали энзиматической амплификации в 35 циклах реакции: 94°C – 5 мин, 62°C – 30 с, 72°C – 1 мин (1 цикл); 94°C – 15 с, 62°C – 15 с, 72°C – 1 мин (4 цикла); 94°C – 5 с, 62°C – 5 с, 72°C – 1 мин (30 циклов). Для выполнения реакций был использован капиллярный термоциклер FTS-1S ("Corbett Research").

Исследование молока трансгенных животных на наличие белка прохимозина и трансформацию его в химозин выполняли методом Western-блот-анализа, заключающегося в иммунологической реакции между иммобилизованным на мембране протеином и специфическим к нему первым антителом с последующей детекцией реакции с помощью видоспецифического маркированного пероксидазой второго антитела, и с помощью биологического теста, основанного на способности химозина осаждать казеины.

Таблица 1. Результаты пересадки микроинъецированных зигот у овец в рамках программы "Генные фермы"

Число реципиентов	25
в т. ч. доноры/реципиенты	20
"чистые" реципиенты	5
Число объягнившихся реципиентов	14
Степень суягности, %	56.0
Число пересаженных зигот	68
Число родившихся ягнят	22
Степень выживаемости зигот, %*	32.4
Число проанализированных ягнят	21
Число трансгенных ягнят	2
Степень трансгенности, %	
от числа родившихся ягнят	9.1
от числа пересаженных зигот	2.9

* От числа пересаженных зигот.

Подготовку проб молока овец для исследования протеинов выполняли следующим образом: 0.5 мл молока смешивали с 0.5 мл кальцийацетатного буфера (10 мМ $CaCl_2$, 100 мМ Na-ацетат, pH 6.0), после чего смесь инкубировали при 4°C в течение 20 мин и центрифугировали 5 мин при 3000*g* для отделения жировой фазы. К оставшейся жидкости (0.8 мл) по каплям добавляли около 300 мкл 0.5 М HCl до pH 2.5. Выпадающие в осадок высокомолекулярные протеины удаляли центрифугированием в течение 5 мин при 13000*g*. Прозрачную надосадочную жидкость инкубировали в течение 90 мин при 30°C для автокаталитического активирования прохимозина. После активации добавлением около 200 мкл 0.5 М NaOH устанавливали pH 6.5 и повторно центрифугировали 5 мин при 13000*g*. Активированные пробы использовали для Western-блот-анализа и постановки биологического теста.

Подготовленные пробы молока трансгенной овцы разделяли электрофорезом в полиакриламидном геле по методу Laemli [13] с 5%-ным концентрирующим и 12%-ным разделяющим гелем с использованием оборудования для вертикального электрофореза (LAB, 10 × 10 см). Перед нанесением в гель образцы смешивали с равным объемом 2-пробного буфера (10 мМ трис-HCl, pH 6,8, 4%-ный SDS, 5%-ный глицерин, 0.01%-ный бромфеноловый синий, 10%-ный 2-меркаптоэтанол) и денатурировали 3 мин при 95°C. В качестве электрофоретического буфера использовали трис-глициновый буфер (25 мМ трис-HCl, pH 8.3, 192 мМ глицин, 0.1%-ный SDS). Электрофорез вели при комнатной температуре при напряжении 100 В для концентрирующего и 120 - 140 В для разделяющего гелей. Транспорт протеинов из геля на нитроцеллюлозную мембрану (BIO-RAD) осуществляли методом полусухого электроблоттинга в течение 90 мин при постоянном токе 90 мА [14]. Для контроля транспорта протеинов на мембрану гель после блоттинга окрашивали раствором Кумаси голубого (0.025% Coomassie Blue R-250, "Sigma"; 40% метанол, "Merk").

С целью блокирования свободных участков связывания неспецифических протеинов мембрану инкубировали 14 - 16 ч в 5%-ном растворе обезжиренного сухого молока в PBS-T (PBS + + 0.1% твин-20).

Источником первого специфического к химозину антитела служила сыворотка кроликов, иммунизированных химозином, которую разбавляли

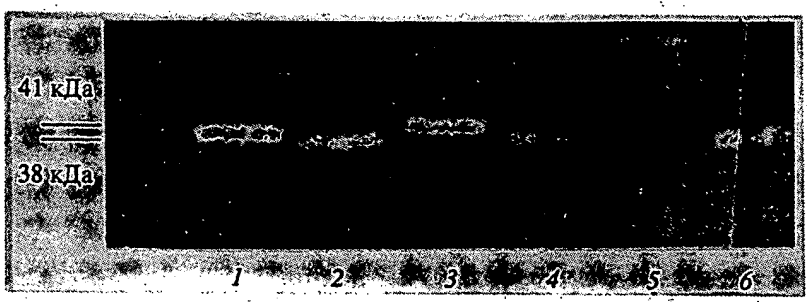

Рис. 2. Western-блот анализ молока трансгенных по генной конструкции baS1Cas–Chym животных. *1, 3* – прохимозин (41 кДа) крупного рогатого скота в молоке трансгенной овцы № 586 и трансгенного кролика № 4382; *2, 4* – активированный при pH 2,5 химозин (38 кДа) крупного рогатого скота в молоке трансгенной овцы № 586 и трансгенного кролика № 4382; *5* – контроль; *6* – химозин фирмы "Sigma".

PBS-T в соотношении 1 : 1000, и с 3 мл такого раствора инкубировали блокированную мембрану при 37°C в течение 4 - 6 ч при постоянном помешивании. Для инкубации использовали 50-мл пробирки ("Falkon"). После этого, трижды промыв мембрану раствором PBS-T, добавляли 3 мл раствора маркированного пероксидазой второго антитела (GtaRb, P-O, "Sigma" : PBS-T = 1 : 1000) и инкубировали 1 - 1.5 ч при 37°C. Для детекции реакции использовали ECL-кит ("Amersham"), в основе которого лежит принцип хемилюминесценции. Мембрану инкубировали 1 мин с детекционным раствором, заворачивали в фольгу ("Saran Folie") с целью предохранения от высыхания и экспонировали на пленку ("Kodak") 5 - 30 с, определяя время экспозиции экспериментальным путем.

Биологическую активность химозина оценивали по времени образования казеинового сгустка коровьего молока при 37°C и сравнением его с калибровочной кривой, построенной на различные концентрации химозина.

После пересадки 68 зигот, микроинъецированных в мужской пронуклеус конструкцией baS1Cas–Chym 25 реципиентам (20 доноры / реципиенты, 5 – "чистые" реципиенты), объягнилось 14 маток (56%), которые принесли 22 ягненка (табл. 1). Две ярки (№ 586, № 596) по результатам PCR-анализа оказались трансгенными (рис. 1). В настоящее время у одной матки (№ 586) установлена передача трансгена потомству: у одного из двух родившихся баранчиков F1 по результатам PCR-анализа было показано наличие трансгена (рис. 1).

Средняя концентрация активного химозина в молоке у лактирующей трансгенной овцы № 586 по результатам 44 анализов составила 292.3 ± 37.1 мг в 1 л. Western-блот молока трансгенной овцы выявил наличие белков ожидаемой массы: 41 кДа (прохимозин) в молоке до активирования и 38 кДа (химозин) в молоке после активирования (рис. 2). Поскольку при низких значениях pH образуется продукт с меньшей молекулярной массой, это определенно указывает на то, что прохимозин трансгенной овцы № 586 способен к аутокаталитическому превращению в фермент химозин. Биологический тест подтвердил неактивность прохимозина и высокую активность химозина, проявляющуюся в способности осаждать казеины молока коров. В предыдущих экспериментах у кроликов, трансгенных по конструкции baS1Cas–Chym, также была зарегистрирована продукция с молоком активного химозина, варьировавшая от 0.1 до 10 г/л [5, 15].

Получение первичных трансгенных по гену прохимозина крупного рогатого скота овец открывает перспективу создания промышленного стада животных – продуцентов с молоком фермента химозина, характеризующегося высокой субстратной специфичностью: 1 г химозина осаждает казеины 10 000 л коровьего молока. Молочная продуктивность овец романовской породы за одну лактацию составляет не менее 100 кг [7]. При необходимости эти животные могут быть скрещены с другими, более молочными, породами овец. Несложные расчеты показывают, что за лактацию одна романовская овца при концентрации химозина в молоке 300 мг/л может дать до 30 г активного фермента. Этого количества химозина будет достаточно для производства 30 т сыра.

При экспрессии химозина в молоко трансгенных животных не требуется выделения химозина в чистом виде, как это необходимо при производстве химозина в дрожжах или экстракции его из сычуга молочных телят, так как он уже находится в молоке.

В последующих исследованиях предполагается размножить трансгенных овец, выявить их потенциальные возможности продуцировать химозин, а также определить качество приготовляемого с его помощью сыра.

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